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INHIBITION OF CHOLESTEROL BIOSYNTHESIS BY A MITOCHONDRIAL EXTRACT[1]

B. B. MIGICOVSKY

Abstract

Cholesterol biosynthesis was effectively inhibited by an extract of mitochondria disrupted with sonic oscillations. The inhibitory effect occurred both in vivo and in vitro. The inhibitor substance was heat-labile, and could be prepared from mitochondria from livers of normal or starved rats. The inhibition appeared to be of the competitive type. The locus of the inhibition in the chain of reactions from acetate to cholesterol appeared to be between acetoacetate and mevalonate. It is suggested that the inhibitor substance present in the mitochondrion represents, in part, the homeostatic mechanism that controls cholesterol synthesis by the liver.

Introduction

Although many of the steps in the biosynthesis of cholesterol have been elucidated, the means by which the body controls this series of synthetic reactions from acetate to cholesterol is still imperfectly understood. Several factors, nutritional, environmental, and physiological, have been shown to exert an effect on cholesterol biosynthesis.

Irradiation of rats with X rays increases the rate of cholesterol synthesis (Gould and Popjak (4)) as do injections of the detergent Triton WR-1339 (Frantz and Hinkelman (3)). Dietary cholesterol decreases the rate of synthesis (Tomkins *et al.* (11)). The importance of the physiological state in cholesterol synthesis is illustrated by the effect of starvation. Hutchens *et al.* (5) showed that the rate of synthesis is decreased in starvation. Migicovsky (7), Migicovsky and Wood (8), Scaife and Migicovsky (9) have shown that the absence of cholesterol synthesis from acetate by liver homogenates from starved rats is in part due to a decreased capacity of the supernate fraction and microsomal fraction to support synthesis, and in part due to the presence of an inhibitor. It has been shown by Migicovsky and Wood (8) that the inhibitor substance resides in the mitochondrial fraction.

The work of Bucher *et al.* (2) suggests that the control mechanism of cholesterol synthesis exerts its effect on a microsome-dependent step in the biosynthetic chain prior to mevalonic acid.

The observations noted above and the studies presented in this paper support the contention that liver cells contain an inhibitor which may be in part the means by which cholesterol synthesis is controlled.

[1]Manuscript received November 5, 1959.
Contribution No. 12, Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario.

Materials and Methods

The liver homogenate preparation and incubation technique, and the technique of measuring the incorporation of acetate and mevalonate into cholesterol were the same as described by Migicovsky (7) and Migicovsky and Wood (8).

Incorporation of acetate-C^{14} into acetoacetate was measured by the method of Stadtman and Barker (10). The mitochondrial extract, which contains the inhibitor, was prepared from the liver homogenate as described by Bucher (1). The homogenate was centrifuged in a Spinco Model L centrifuge at 9000 $\times$ *g*. The residue was suspended in water equal in volume to the original volume of homogenate and subjected to sonic oscillations for 15 minutes. The resulting homogenate of mitochondria was centrifuged at 100,000 $\times$ *g*. The supernate was decanted and lyophilized. When required, the lyophilized supernate containing the inhibitor substance was dissolved in the same buffer used in preparing the original homogenate, and an appropriate quantity added to the incubation mixture. The duration of incubation was 2 hours unless otherwise specified.

The rats used in the experiments weighed from 110–135 g. They were either normally fed, or starved for 48 hours before they were killed and their livers excised. In the subsequent discussion the terms "normal" and "starved" are used to denote homogenate fractions prepared from livers of normally fed or starved rats.

Data shown in Table I were obtained from liver homogenates of rats killed 3 hours after an intraperitoneal injection of either 0.5 ml buffer or 0.5 ml aqueous solution containing 30 mg dried mitochondrial extract. Data given in Table IV were obtained from liver homogenates of normal or starved rats killed 2 hours after an intraperitoneal injection of either 1.12 μmoles mevalonic acid-2-C^{14} or 2.57 μmoles acetate-1-C^{14}.

Results and Discussion

The inhibitory effect of mitochondria from livers of starved rats on in vitro cholesterol synthesis by a liver homogenate system prepared according to Bucher (1) was reported in an earlier publication (8). Figure 1 shows that the inhibitory effect is also exerted by the mitochondrial extract. The experiment was carried out with a Bucher homogenate serving as the synthetic system, to which varying quantities of inhibitor preparation were added. The data in Table I demonstrate that a similar inhibitory effect was induced in liver homogenates prepared from rats injected with mitochondrial extract.

The data in Table II show that when a constant amount of mitochondrial extract was added to the incubation mixture, an increase in the concentration of synthetic system ("normal" homogenate) resulted in a progressive reduction in the percentage of inhibition. A doubling of the substrate (acetate) appeared to have no significant effect. Figure 2, however, demonstrates that when the amount of homogenate, with or without inhibitor, was kept constant, and the amount of substrate was progressively increased, the amount of incorporation progressively decreased. Reciprocals of substrate and of acetate incorporated

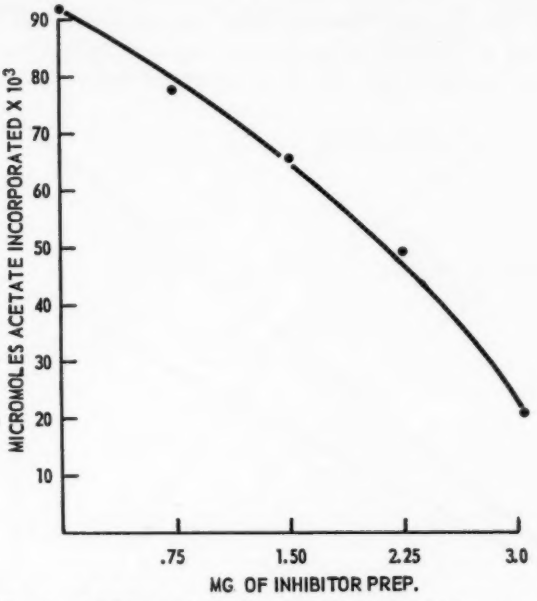

FIG. 1. Inhibitory effect of the dried mitochondrial extract.

TABLE I

Effect of intraperitoneal injection of inhibitor substance on in vitro acetate incorporated into sterol by liver homogenate

Acetate incorporated, μmoles × 10^3	
Homogenate from inhibitor treated rat	Homogenate from rat injected with buffer
18.0	39.1
6.9	30.0
5.1	11.0

TABLE II

Inhibition by mitochondrial extract of acetate incorporated into sterol by varying quantities of liver homogenate

Amount of liver homogenate,* ml	% inhibition	
	1.56 μmoles acetate	3.12 μmoles acetate
1.00	94.5	96.1
1.25	88.8	88.9
1.50	23.9	27.9
2.00	15.3	12.8

*Constant amount (1.5 mg) mitochondrial extract added to incubation mixture.

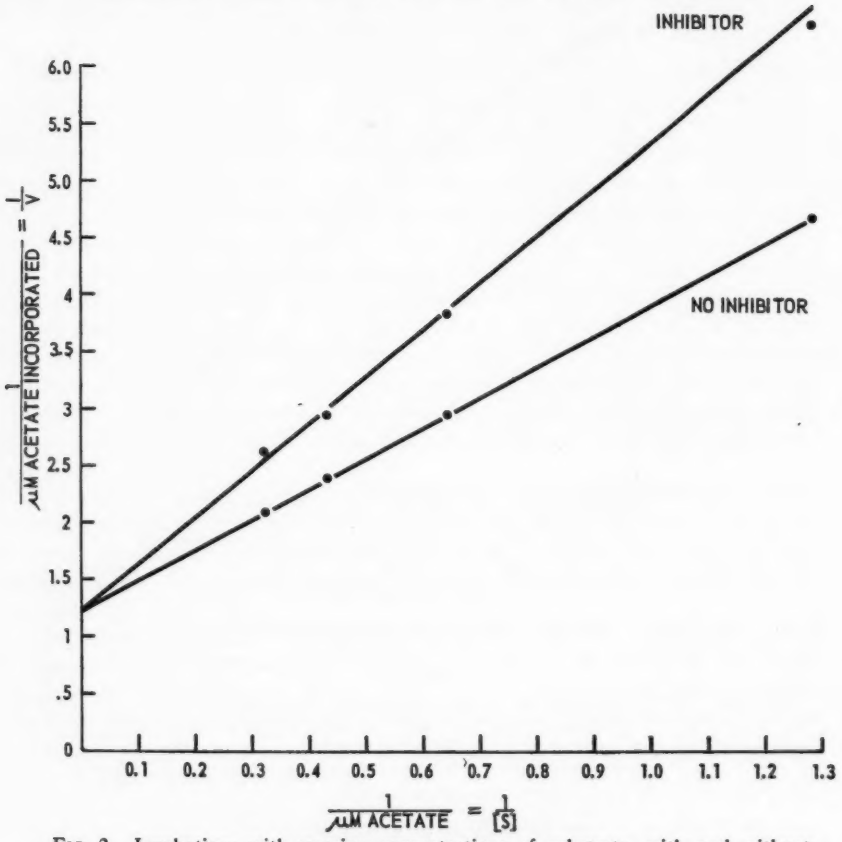

FIG. 2. Incubations with varying concentrations of substrate, with and without inhibitor.

into cholesterol are shown on the axes (Lineweaver and Burk (6)). These results indicate that the inhibitor present in the mitochondrial extract is of the competitive type. Results given in Table III indicate that the inhibitory substance is heat-labile.

TABLE III

Lability of inhibitor substance to heat

Addition to incubation mixture	Acetate incorporated, μmoles × 10^3	
	Trial 28	Trial 29
None	65.4	91.5
Inhibitor (2 mg)	44.1	20.9*
Boiled inhibitor	64.9	83.8
Supernate from boiled† inhibitor solution	67.2	90.4

*In trial 29, 3 mg of inhibitor was used.
†The 1.0% solution of inhibitor was boiled for 1 minute and spun down at 40,000 r.p.m. yielding a clear supernate.

Figure 3 shows that extracts from both "normal" and "starved" mitochondria have an inhibitory effect, and that the degree of inhibition is greater with the "starved" extract. It was observed earlier (8) that whole mitochondria from

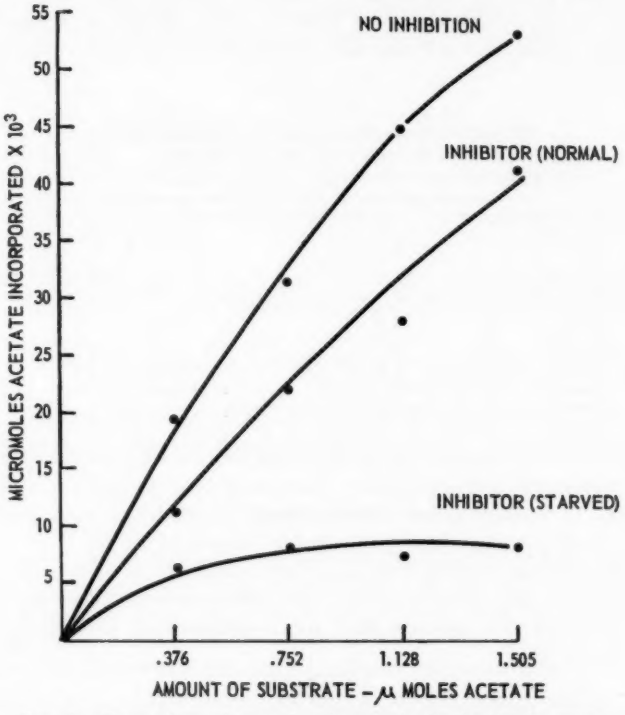

FIG. 3. Comparison of normal and starved mitochondrial extracts.

"normal" livers, unlike those from "starved" livers, did not inhibit cholesterol synthesis. It might be concluded, therefore, that in starvation not only is the activity (or amount) of inhibitor increased but the permeability of the mitochondrion to it is also increased.

Table IV gives a comparison of the amount of cholesterol synthesized in vivo from acetate and from mevalonate injected into normal and into starved rats. Each value shown represents the mean of four individual determinations.

TABLE IV

In vivo incorporation of acetate and mevalonate into liver cholesterol

	% C^{14}-acetate incorporated	% C^{14} mevalonate incorporated
Normal rats	0.34	22.9
Starved rats	0.055	24.6

The data indicate, and statistical analysis confirmed, that starvation caused a marked reduction in the synthesis of cholesterol from acetate, but not from mevalonate. The locus of inhibition in the chain of reactions between acetate and cholesterol thus appears to be between acetate and mevalonate.

The same interpretation may be placed on data shown in Table V. Liver homogenates prepared from starved rats do not synthesize cholesterol from

TABLE V

In vitro incorporation of acetate and mevalonate into cholesterol by liver homogenate

	μmoles acetate × 10^3	μmoles mevalonate × 10^3
Normal	4.5	77.3
Starved	0	13.6
Normal	12.5	91.5
Starved	0	77.6
Normal	6.8	91.3
Starved	0	47.2
Normal	4.0	172.0
Starved	0	120.0

acetate (8) but the "starved" homogenate preparation did incorporate mevalonate into cholesterol, although not to the extent that occurred with "normal" homogenate. In another series of tests, whole mitochondria from the liver of a starved rat and mitochondrial extract were added to different aliquots of a "normal" homogenate preparation, and their inhibitory activity was measured with acetate-1-C^{14} and mevalonate-2-C^{14} as substrates for cholesterol synthesis. The data in Table VI indicate that the mitochondrial suspension and the

TABLE VI

In vitro inhibition by mitochondrial preparations of incorporation of acetate and mevalonate into cholesterol

Addition to 2.0 ml normal homogenate	μmoles acetate incorporated × 10^3	Inhibition, %	μmoles mevalonate incorporated × 10^3	Inhibition, %
None	12.5		91.5	
Starved mitochondria*	2.7	78.4	52.2	43.0
None	6.8		91.3	
Starved mitochondria*	2.1	69.1	87.0	4.7
None	10.4		93.5	
Mitochondrial extract†	5.8	44.2	89.5	9.1
None	5.0		80.1	
Mitochondrial extract†	1.2	76.0	55.7	30.5

*The amount of mitochondria added was equivalent to one-third of that present in normal homogenate.
†3.0 mg was added in 0.2 ml buffer.

mitochondrial extract behave in a similar manner with respect to the nature of the substrate. Cholesterol synthesis was markedly inhibited with acetate as the substrate whereas inhibition was significantly less with mevalonate.

The inhibition of cholesterol synthesis from acetate is not accompanied by any change of incorporation of the acetate into acetoacetate. A mitochondrial suspension was added to a normal homogenate and the inhibitory effect was measured with both acetate and mevalonate as substrates. Incorporation of C^{14} into the carboxyl carbon of acetoacetate was also measured. The data are shown in Table VII and it is apparent that marked inhibition of cholesterol

TABLE VII

Effect of inhibitory mitochondria on incorporation of acetate and mevalonate into acetoacetate and cholesterol

Addition to 2.0 ml normal homogenate	μmoles acetate incorp. × 10^3		μmoles mevalonate incorp. × 10^3	
	Acetoacetate†	Cholesterol	Acetoacetate†	Cholesterol
None	63.8	9.5	1.22	112.1
"Starved" mitochondria*	61.7	4.3	1.37	100.4

*The amount of mitochondria added was equivalent to one-third of that present in the normal homogenate.
†The value given is for μmoles acetate or mevalonate incorporated into the carboxyl carbon of acetoacetate.

synthesis occurred when acetate was the substrate and only slightly, if at all, when mevalonate was the substrate. The mitochondrial suspension did not affect the incorporation of either acetate or mevalonate into acetoacetate. Also, very little of the C^{14} from mevalonate-2-C^{14} was incorporated into acetoacetate.

Conclusions

The foregoing experiments indicate that an inhibitor to cholesterol synthesis, heat-labile and water-soluble, is produced within the mitochondrion. The inhibition appears to be of the competitive type and is active between acetoacetate and mevalonate. These observations explain in part why, in the starvation state, cholesterol synthesis is affected so drastically. The inhibitor substance is present in the liver mitochondria of both normal and starved rats, but apparently is not released as readily by "normal" mitochondria as by "starved" mitochondria.

It is possible that this material represents in part the homeostatic mechanism which controls cholesterol production by the liver. It would indeed be of interest to investigate whether X-irradiation or Triton, which overcome the fasting effect (2), would also counteract the inhibitory effect of mitochondrial extract.

Acknowledgments

I would like to acknowledge the technical assistance of F. Pacha and the staff of the Small Animal Laboratory, Animal Research Institute, Ottawa, Ontario.

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THE ACTION OF PANCREATIC DEOXYRIBONUCLEASE ON OLIGONUCLEOTIDES FROM CALF THYMUS DEOXYRIBONUCLEIC ACID[1]

R. O. Hurst and Dorothy Findlay

Abstract

Hydrolysis of sodium oligonucleotide by crystalline pancreatic deoxyribonuclease (DNA-ase) has been studied in the presence of different metal ions and the chelating agent ethylenediaminetetraacetate (EDTA). Although EDTA inhibited the action of DNA-ase when magnesium or cobaltous ions were used as activator, the enzyme activity was enhanced in the presence of manganous ions and EDTA. The results are interpreted as indicating the presence of an oligonucleotidase function in the enzyme preparation. A differential assay method for DNA-ase and oligonucleotidase activity has been devised and the evidence obtained gives additional support for this conclusion.

Introduction

Previous investigation of the phosphodiesterase activity of pancreatic deoxyribonuclease (DNA-ase) by Hurst (1) has shown that the hydrolysis of calf thymus deoxyribonucleate (DNA) to products soluble in the uranyl acetate reagent of Hurst, Little, and Butler (2) is considerably increased in the presence of high concentration of the enzyme. The existence of two enzymic functions in DNA-ase was suggested as an explanation of the results obtained. This report presents the results of further studies on the functional heterogeneity of DNA-ase. The use of sodium oligonucleotide as substrate has led to the adoption of the name 'oligonucleotidase' to describe the enzymic function that is responsible for additional hydrolysis of limit oligonucleotides.

Materials and Methods

Deoxyribonucleic Acid

This was prepared from calf thymus by the method of Hurst (3). The preparation used in this work had the following properties: nitrogen:phosphorus ratio, 1.69; specific viscosity of aqueous solution per gram of phosphorus per liter, 64; extinction coefficient as absorbance per mole of phosphorus per liter at 2600 Å, 8880, and at 2300 Å, 3500. The yield was 15 g of nucleate from 700 g of minced tissue.

Sodium Oligonucleotide

Five grams of DNA was dissolved in 200 ml of water containing sodium ethyl mercurithiosalicylate (merthiolate) at a final concentration of 0.0125%. The mixture was allowed to stand at 5° C overnight to complete the dissolving of the nucleic acid. Magnesium acetate (0.20 *M*) was added to give a final concentration of 0.003 *M* and the volume adjusted to 250 ml with water. Two milliliters of a solution of crystalline DNA-ase (1 mg/ml) was added dropwise with stirring over a 5-minute period. After 6.5 hours at room temperature the

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Contribution from the Department of Biochemistry, Queen's University, Kingston, Ontario. This work was supported by a grant from the National Research Council of Canada.

pH of the enzymic digest was increased to 8.0 with 0.1 *N* sodium hydroxide solution and the sodium oligonucleotide was precipitated by addition to four volumes of 95% alcohol saturated with sodium acetate. In subsequent studies it was found preferable to concentrate the preparation *in vacuo* at 30° C to 50 ml before precipitation with alcohol. The mixture was stored at 5° C overnight; the supernatant solution was removed by decantation and the precipitate recovered by centrifugation at 450 × *g*. The precipitate was washed twice with 50 ml of 95% alcohol, then with absolute alcohol, and finally with ether, by centrifugation. The sodium oligonucleotide was dissolved in a minimum of water, and, after any insoluble material had been removed by centrifugation, the supernatant solution was lyophilized. The yield of white, powdery material was 4.2 g. Analysis showed that 95% of the total phosphate was soluble in 10% trichloroacetic acid and that 6.9% was soluble in the uranyl acetate reagent. The nitrogen:phosphorus ratio was 1.71. These analyses indicate that this oligonucleotide material is comparable to the magnesium oligonucleotide described by Little and Butler (4) as limit oligonucleotides.

Deoxyribonuclease

Once crystallized DNA-ase (lot numbers: D-575, D-630, D-660, D-672, and 650-B) was obtained from the Worthington Biochemical Corporation, Freehold, New Jersey. Lots D-575, D-630, and 650-B were crystallized by the method of Kunitz (5), but lots D-660 and D-672 were prepared by the same isolation procedure with the added modification that the crystalline material was redissolved and dried from solution, according to a report received from the supplier (6).

Crude DNA-ase A was a lyophilized product, prepared according to the method of McCarty (7) and generously supplied by Dr. G. C. Butler.

Crude DNA-ase B was prepared by a modification of the method of McCarty (7), using centrifugation at 5° C in place of filtration. This enzyme preparation melted during the process of lyophilization and was accordingly refrozen to complete the process.

Phosphorus Determinations

Total phosphorus determinations were made by a modification of the method of Beveridge and Johnson (8) as described by Hurst (1). Acid-soluble phosphorus (ASP) and uranyl-acetate-soluble phosphorus (USP) determinations were carried out as reported by Hurst (1).

Use of Uranyl Acetate Reagent for Determination of Soluble Nucleotide Material

MacFadyen (9) originally suggested a uranyl chloride reagent for following ribonuclease activity, and a modification of this method was used by Hurst, Little, and Butler (2) in a study of phosphodiesterase activity on magnesium oligonucleotide. The digestion of DNA with DNA-ase leads to the formation of both ASP and USP material. Under the conditions employed it has not been possible to define the solubility limits of the low molecular weight oligonucleotides in the uranyl acetate reagent. Holden and Pirie (10) observed that nucleotide concentration of the digestion products of yeast ribonucleic acid in their uranyl nitrate – trichloroacetic acid reagent was a determining factor for

solubility. The data in Table I indicate that, in order to use solubility of nucleotide material in the uranyl acetate reagent as a criterion for enzymic

TABLE I

The effect of concentration on the solubility of oligonucleotide phosphorus in uranyl acetate reagent

Oligonucleotide phosphorus, μg per ml of sample	Dilution factor	USP as % total phosphorus
146.0	—	10.6
109.5	1.3	10.1
73.0	2	14.3
36.5	4	24.6
14.6	10	51.1

degradation, the sampling procedure must be standardized. For this reason all enzymic digests were carried out at 3.12 mg of sodium oligonucleotide per ml, which gave a phosphorus concentration of about 200 μg per ml of sample.

Hydrolysis of Sodium Oligonucleotide by DNA-ase

The enzymic digests used for metal ion activation studies were similar to those described by Hurst (1) with the exception that Tris (tris(hydroxymethyl)aminomethane)–acetate buffers were used in place of Tris–hydrochloride buffers. The substrate solution was prepared by dissolving sodium oligonucleotide in 0.10 *M* Tris–acetate buffer to give a solution of 5.0 mg per ml. The desired final concentration of metal ion was achieved by addition of the appropriate volume of 0.20 *M* magnesium acetate, manganous acetate, or cobaltous chloride. When ethylenediaminetetraacetate (EDTA) was required in the enzymic digest, it was necessary to make up a solution of the metal salt and EDTA and, before addition, to adjust the pH to that used in the buffered substrate solution. The experimental procedure was the same as that reported previously (1) and the time course of hydrolysis was determined by estimation of USP as a percentage of the total phosphate.

Differential Assay Method for DNA-ase

The activity unit for hydrolysis of DNA by DNA-ase is defined as the amount of enzyme that catalyzes the formation of 1% ASP per hour under the conditions for the assay procedure. Specific activity is expressed as DNA-ase units per mg of protein. For the assay procedure, 25.0 mg of DNA was dissolved in 5.0 ml of 0.10 *M* Tris–acetate buffer, pH 7.0, and 0.2 ml of 0.5% merthiolate was added. The solution was allowed to stand at room temperature for a few hours and then stored at 5° C until the DNA was completely dissolved. All such solutions were used within a week. Immediately before use 0.8 ml of 0.20 *M* magnesium acetate was added and the volume adjusted with water to 7.0 ml. This substrate solution was equilibrated in a water bath at 37° C for 10 minutes. At zero time 1.0 ml of the enzyme solution was added and samples were taken exactly at 15 minutes for determination of total phosphorus and

ASP. If the activity of the enzyme was such that the ASP was higher than 50% it was necessary to dilute the preparation accurately and introduce a dilution factor in the calculation of the specific activity.

The activity unit for the hydrolysis of sodium oligonucleotide by the enzyme preparation is defined as the amount of enzyme that catalyzes the formation of 1% USP per hour under the conditions for the assay procedure and the specific activity as units per mg of protein. In this method 2.5 ml of a 0.5% solution of sodium oligonucleotide in 0.10 *M* Tris–acetate buffer, pH 8.0, 0.1 ml of 0.5% merthiolate, and 0.1 ml of 0.20 *M* manganous acetate:0.10 *M* EDTA, pH 8.0, were combined and the volume adjusted to 3.0 ml with water. After equilibration at 37° C for 10 minutes, 1.0 ml of the enzyme solution was added. Samples for total phosphorus and USP were taken exactly at 30 minutes after addition of enzyme and the difference between the percentage of USP at 30 minutes and at zero time was determined and used in the calculation of the specific activity.

Results and Discussion

Several experiments were carried out to determine the effect of EDTA on the activation of the enzymic hydrolysis of sodium oligonucleotide by magnesium and manganous ions but only one series is presented in Tables II and III. In all cases it was found that higher values for USP were obtained in the

TABLE II

The effect of EDTA on the hydrolysis of sodium oligonucleotide by DNA-ase in the presence of magnesium acetate and TRIS–acetate buffer (0.0625 *M*) at pH 8*

Mg^{++} (*M*)	EDTA (*M*)	Unchelated EDTA†	USP as % total P		
			2 hr	24 hr	48 hr
0.0125	—	—	26.3	46.8	51.3
0.0200	—	—	28.0	43.7	53.5
0.0250	—	—	27.4	44.6	54.0
0.0250	0.0125	5.0×10^{-7}	16.4	25.0	25.9
0.0400	0.0200	8.0×10^{-7}	17.5	26.7	28.9
0.0500	0.0250	10.0×10^{-7}	14.1	26.7	29.0

*Each digest contained 3.12 mg sodium oligonucleotide per ml and 0.125 mg of DNA-ase (Worthington 1X, lot D-630) per ml.
†Calculated from data reported by Chabarek, Bersworth, and Martell (11).

TABLE III

The effect of EDTA on the hydrolysis of sodium oligonucleotide by DNA-ase in the presence of manganous acetate and TRIS–acetate buffer (0.0625 *M*) at pH 8*

Mn^{++} (*M*)	EDTA (*M*)	Unchelated EDTA†	USP as % total P		
			2 hr	24 hr	48 hr
—	—	—	10.8	13.5	13.4
—	0.00250	—	5.4	5.4	5.4
0.00125	—	—	25.5	48.6	50.1
0.00250	—	—	27.5	50.0	54.9
0.00375	—	—	26.9	50.2	54.6
0.00250	0.00125	9.9×10^{-13}	32.7	58.8	62.2
0.00500	0.00250	2.0×10^{-12}	33.3	58.3	62.0
0.00750	0.00375	3.0×10^{-12}	32.1	60.0	64.5

*Each digest contained 3.12 mg sodium oligonucleotide per ml and 0.118 mg of DNA-ase (Worthington 1X, lot D-630) per ml.
†Calculated from data reported by Chabarek, Bersworth, and Martell (11).

presence of the manganous – EDTA complex and that EDTA inhibited the magnesium-activated enzymic reaction. The stability constant (log K) for the manganous – EDTA complex at pH 8.0 is 16.2 as calculated from the data of Chabarek, Bersworth, and Martell (11). This may be compared with an approximate value of log K equal to 3.6 for the stability constant of the enzyme – manganese complex, calculated from the data in Table III on the assumption that the rate of the enzymic reaction is determined by the amount of metal – enzyme complex present in the enzyme digestion mixture. The fact that, despite this disproportion in the values of the formation constants, the rate of the enzymic reaction in the presence of EDTA and manganese is enhanced suggests that the manganous – EDTA complex is capable of combining with enzyme or with substrate.

Since manganese is a transitional element, six orbital electron levels are available for complex formation, four of which could be filled by EDTA, leaving two for combination with imidazole or carboxyl groups of the enzyme or with phosphoryl groups of the substrate. Such a differential competition for the metal activator may provide an explanation for the enhancement of activity in the presence of EDTA and manganous ions.

Although cobalt is also a transitional element, the activation of DNA-ase by cobaltous ions was inhibited in the presence of EDTA (Table IV). It is

TABLE IV

The effect of EDTA on the hydrolysis of sodium oligonucleotide by DNA-ase in the presence of cobaltous chloride and TRIS–acetate buffer (0.0625 M) at pH 6.8*

Co^{++} (M)	EDTA (M)	Unchelated EDTA†	USP as % total P		
			2 hr	24 hr	48 hr
0.015	—	—	32.6	57.8	67.7
0.020	—	—	36.6	59.2	66.0
0.025	—	—	28.9	56.9	65.0
0.015	0.0075	2.4×10^{-12}	28.0	49.2	56.8
0.030	0.015	4.8×10^{-12}	25.7	47.5	52.9
0.040	0.020	6.4×10^{-12}	26.6	40.5	49.5
0.050	0.025	8.0×10^{-12}	22.1	34.9	42.6

*Each digest contained 3.12 mg sodium oligonucleotide per ml and 0.125 mg of DNA-ase (Worthington 1X, lot D-630) per ml.
†Calculated from data reported by Chabarek, Bersworth, and Martell (11).

therefore apparent that the increased activation of DNA-ase by manganous ion in the presence of EDTA is not a general effect of the transitional elements. Mounter, Floyd, and Chanutin (12) have found that manganous ion activation of dialkylfluorophosphatase is increased in the presence of histidine but cobaltous ion activation is inhibited. Activation of DNA-ase by histidine has been reported by Nemchinskaya and Shapot (13).

The unusual effects of metal ion binding on DNA-ase activity may be taken as implicating an imidazole center in the phosphodiesterase function of this enzyme. The suggestion by Wiberg (14) that the metal ion complex is necessarily formed only with the substrate on the basis of the independence of activator and enzyme concentration is not valid if the heterogeneity of DNA-ase is considered since this conclusion is based on studies involving the determina-

tion of the initial activity of the enzyme on DNA. The fact that the stability constant for manganous – DNA derived from data provided by Wiberg and Neuman (15) is of the same order (log K equal to 3.5) as for the enzyme – manganese complex indicates that the enzyme and substrate may successfully compete for the metal ion. Since secondary phosphate groups are liberated by the action of the enzyme, the metal ion requirement for the enzyme might very well be limited by such a competition. This may provide an explanation for the rapid decrease in the initial rate of the enzymic reaction. The need for excess manganese over EDTA is evidence that free manganous ion plays a role in addition to that of the manganous – EDTA complex. This is supported by the fact that 38% inhibition was obtained when both EDTA and manganous ion were present at 0.0125 M concentration.

The use of manganous ion and EDTA has made it possible to increase the liberation of material soluble in the uranyl acetate reagent by the action of DNA-ase on DNA to a rate considerably higher than that reported previously by Hurst (1). The concentration of DNA-ase required for maximal activity is indicated by the data in Fig. 1. The highest value obtained was 92% USP in

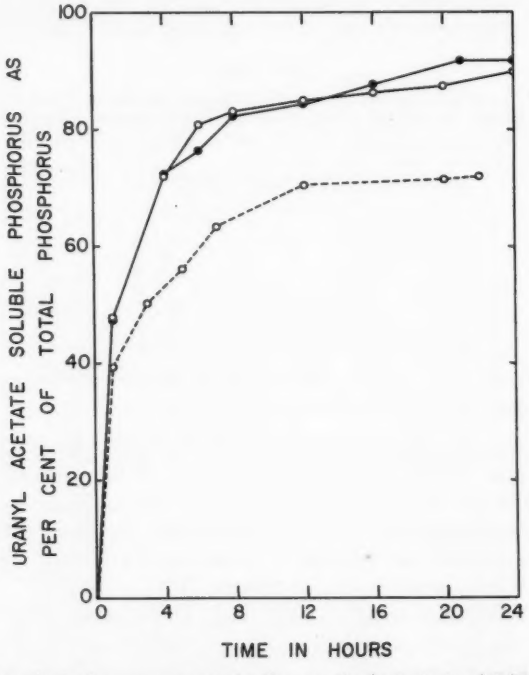

FIG. 1. The effect of enzyme concentration on the hydrolysis of DNA (2.0 mg/ml) by DNA-ase in the presence of 0.0075 M manganous sulphate and 0.0045 M EDTA in 0.06 M Tris–acetate buffer at pH 8 and 37° C. – – – ○ – – –, 0.12 mg DNA-ase per ml; ——●——, 0.24 mg DNA-ase per ml; ——○——, 0.34 mg DNA-ase per ml. Enzyme preparation used: Worthington 1X, lot D-575.

24 hours. Analysis for inorganic phosphate in all digests at 24 hours showed none present.

The results of this study provide additional evidence for the presence of another enzymic function in DNA-ase. Since this phosphodiesterase function is capable of catalyzing the further hydrolysis of oligonucleotides, which were obtained from DNA under conditions previously considered to permit the completion of its action, the term oligonucleotidase may be used to describe it. Variations in the relative amounts of oligonucleotidase activity in the crystalline enzyme preparations were first noted when it was found that lot D-660 gave very low values for USP in the presence of magnesium ion and EDTA (7.7% USP for 0.020 *M* magnesium acetate:0.010 *M* EDTA in 48 hours as compared with 17.1% USP in the absence of EDTA) in contrast to those reported in Table II for lot D-630. The results of a differential assay method using DNA as substrate for the estimation of DNA-ase activity and sodium oligonucleotide as substrate for the estimation of oligonucleotidase activity are presented for several enzyme preparations in Table V. It is apparent that crystallization of

TABLE V

DNA-ase and oligonucleotidase activities of several enzyme preparations

Enzyme preparation	DNA-ase		Oligonucleotidase		Ratio of DNA-ase to olig-ase activity
	Specific activity (units/mg)	Yield*	Specific activity (units/mg)	Yield*	
Crystalline enzyme					
Lot D-575	—	—	80.2	157	—
Lot D-630	32,400	112	51.6	101	627
Lot D-660	28,500	98	41.2	80	691
Lot D-672	25,900	89	37.2	73	695
Lot 650-B	29,000	100	51.2	100	570
Lot 650-B†	26,600	92	31.0	61	860
Crude DNA-ase A	2,050	7	15.5	30	132
Crude DNA-ase B	4,380	15	3.5	7	1,250

*Yield is expressed as a percentage of the activity obtained for lot 650-B.
†Enzyme solution frozen and thawed before assay was made.

the enzyme has brought about a 10- to 15-fold increase in DNA-ase activity but only a 2- to 5-fold increase in oligonucleotidase activity over that found for the Crude DNA-ase A preparation. In addition it is noted that lot D-575, which was the first preparation of crystalline DNA-ase obtained from Worthington Biochemical Corporation in this work, was the most active with respect to the specific activity for oligonucleotidase. When it was discovered that preparations D-660 and D-672, which showed a drop in oligonucleotidase activity of 77% and 84% when compared with D-575, were isolated by a modified procedure that involved an additional step of redissolving the crystalline material and lyophilization, preparation 650-B was obtained in which this step was eliminated. Assay of lot 650-B before and after freezing and thawing showed that whereas the DNA-ase activity only decreased by 8%, the loss of oligonucleotidase activity was 39%. In the preparation of Crude DNA-ase B, the refreezing of the enzyme solution during the lyophilization step is the most

probable factor for explaining the observation that this enzyme preparation resulted in the lowest yield of oligonucleotidase activity. It is accordingly apparent that the oligonucleotidase function in DNA-ase is much less stable to the denaturative forces involved in the process of freezing and thawing the protein solution. The marked variations obtained in the ratios of the specific activities for DNA-ase and oligonucleotidase functions suggest that the microheterogeneity that has been observed is due to the presence of separate protein entities.

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ZONE ELECTROPHORESIS OF CALF THYMUS HISTONE IN STARCH GEL[1]

J. M. NEELIN[2] AND E. M. NEELIN[3]

Abstract

A total of 19 zones were detected by starch gel electrophoresis of calf thymus histone in buffers of 0.02 μ below pH 5.0. Of these, 18 were evident at pH 4.9 (acetate) and 15 at pH 3.9 (formate). Above pH 5.0, aggregation interfered with resolution, but by adding 4 *M* urea to the gel sufficient resolution was obtained between pH 4.4 and 8.7 to distinguish a total of 22 zones. Of this total, three fast components were present only occasionally in trace amounts, and three others were resolved from the immobile aggregated histone at low pH only. At least 16 zones appeared to be native histone components. Acid extraction of the histone did not appear to cause degradation since no new N-terminal amino acids were generated by this step. Two different methods of preparation produced histone extracts with essentially the same electrophoretic properties.

Introduction

The presence of 16 components in chicken erythrocyte histone has been demonstrated by starch gel electrophoresis (SGE) (1). Since the number of components resolved by this technique exceeds that observed with any other method, SGE was applied in the present study to calf thymus histone and its chromatographic fractions. The possible contribution of aggregates and hydrolytic products to the observed number of components was examined with histone prepared by different methods and with N-terminal amino acid assays. The possible occurrence of artifacts peculiar to the method was examined by performing electrophoresis in different solvents, and by confirming the consistency of the resolution by a second SGE of electrophoretic fractions. This study substantiated the complexity of histone and verified the sensitive resolving power of the technique when applied to this protein.

Methods

Calf thymus histone was prepared in two ways, by extraction at pH 1.7 from nuclei isolated in dilute citric acid (2), and by extraction at pH 1.7 from whole tissue without prior isolation of nuclei. In the latter method a portion of the nuclear-rich gland was vigorously blended in an equal weight of water, adjusted to pH 1.7 before and after blending. After centrifugation at 1100 $\times$ *g* for 15 minutes, the supernatant solution was adjusted to pH 4, one volume of 8 *M* urea was added, and any further precipitate was removed by centrifugation for 30 minutes at 5000 $\times$ *g*. This solution was of sufficient concentration for satisfactory zone electrophoresis.

Smithies' (3) method of zone electrophoresis in starch gel was used. 'Starch—Hydrolyzed for gel electrophoresis' was obtained from Connaught Medical

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[2]National Research Council Postdoctorate Fellow, 1959.
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Laboratories, Toronto; 16 g of starch (3 g more than recommended for serum) was dispersed in 100 ml of buffer (formate, acetate, cacodylate, or barbiturate solutions). Since no differences were observed when the bridge solutions were sodium chloride, formate, or acetate buffers of ionic strength 0.15, unbuffered sodium chloride was used for convenience at all pH values. All electrophoretic analyses were made at a constant current of 3.5 ma per cm of gel width, which required a voltage gradient of approximately 3.0 v per cm for gels containing buffer solutions only, and 2.5 v per cm for buffered urea solutions. Constant temperature is required for reproducible resolution; maintenance of constant current and the use of water-soaked wicks across the trays was adequate for this purpose. Usually the two faces of a sliced gel were respectively stained with amido black 10B and nigrosin (Hartman-Leddon Co.), dissolved in the solution for removal of excess stain (methanol – acetic acid – water, 5 :1:5 by volume). The use of rinses of absolute methanol after the first washing reduced fading of the pattern stained with amido black.

Electrophoretic fractions were subjected to a second SGE as follows: after electrophoresis and slicing of the gel, one slice was stained and used as a guide for the cutting of 4-mm sections from the other slice. These sections were each inserted in moistened slots at the origin of a wide (12 cm) gel, and analyzed in parallel with a sample of the original histone solution.

The chromatographic fractions were obtained by cation-exchange chromatography on the sodium form of Amberlite IRC-50 (2). The 'lysine-rich histone' (three successive fractions from peak sL (2)), and the 'arginine-rich' components (peaks sA_1 and sA_3), were dialyzed to diminish the concentration of eluent salts, centrifuged, and subjected to zone electrophoresis in a single gel in parallel with a sample of whole histone.

Gels in urea were made by rapidly adding starch to a solution of buffered 4 *M* urea. Gels with 7 *M* urea were too fragile for convenient manipulation. As the pH of the dilute buffer solutions changed with the addition of starch and again on the addition of urea, all pH values quoted were measured on the gel.

Segments of gel containing 4 *M* urea were transparent. These were scanned at 0.5-mm intervals in a Beckmann DU spectrophotometer (slit width of 0.5 mm) fitted with a Perspex-and-quartz cell (55×20×6 mm). Diffusion of the protein was not significant during the scanning period, and the segments were subsequently sliced and stained.

N-terminal amino acids were determined by Phillips' (4) modification of the 1-fluoro-2,4-dinitrobenzene (FDNB) method (5). Hydrolyses of dinitrophenyl-histone (DNP-histone) were carried out either in 11 *M* hydrochloric acid in a sealed bulb at 100° for 4.5 hours (to minimize breakdown of labile DNP-amino acids), or by refluxing for 24 hours in 5.5 *M* hydrochloric acid (to break resistant peptides). In one determination the histone was reacted with FDNB in two volumes of methanol in accordance with Luck *et al.* (6). Washed nuclei were reacted in methanol to reduce viscosity due to nucleic acid. Paper chromatographic separation and quantitative measurement of DNP-amino acids utilized the steps outlined by Smith and Turner (7). Yields were expressed

as micromoles of DNP-amino acids, corrected by factors reported by Phillips (4).

Results

Starch Gel Electrophoresis

Freshly extracted histone was subjected to SGE under varied conditions of pH, ionic strength, and protein concentration. As with chicken erythrocyte histone (1), migration was directly dependent on ionic strength between 0.005 and 0.050 despite the reduced voltage necessary to maintain constant current. At ionic strength below 0.03, resolution was sharpened and mobilities became dependent on the protein concentration (probably because of its significant ionic contribution). At concentrations of whole histone extract in excess of ca. 0.6 mg of histone nitrogen per ml, the more concentrated zones tended to flood over adjacent leading zones.

The decrease in mobility with increasing pH is illustrated in Fig. 3, which gives examples of patterns obtained at pH 3.9 in formate buffer, pH 4.9 and pH 5.4 in acetate buffer, and pH 6.3 in cacodylate buffer, all at ionic strength 0.020. As fine gradations in contrast are lost by photography and reproduction, diagrams of the patterns observed in the original gels are included with each photograph. Zones are numbered in order of decreasing mobility to facilitate reference,* but are not to be identified with the numbers assigned to chicken erythrocyte histone zones (1). In the various patterns, comparable zones were identified by their relative mobilities and staining intensities; the identification was facilitated by a metachromatic staining property of this amido black sample with histone. Most zones stained the usual grey-blue, but zones (*3*) to (*7a*), and (*8*) appeared a clear turquoise, while (*15*) and (*16*) were a mixture of the two shades. The identity of similar zones under different conditions must be considered tentative, particularly for zones present in small amounts. Fifteen zones were identified at pH 3.9, and 18 at pH 4.9, the additional components appearing mainly in the region of zones (*6*) to (*10*). Above pH 5.2 mobilities of most components decreased sharply and resolution of zones was largely abolished by the formation of aggregates (2), which penetrate the gel slowly. The most effective conditions for analysis appeared to be pH 4.9 in acetate buffer, ionic strength 0.02; this ionic strength was used in all electrophoreses to be described subsequently. Under these conditions many different preparations from isolated calf thymus nuclei have produced electrophoretic patterns differing from that in Fig. 3 only occasionally and slightly. For example the amounts of zones (*1*) and (*2*) were frequently insufficient to produce significant staining at histone concentrations suitable for other zones, and disappeared together with (*3*) and (*4*) on dialysis. Zones (*17*), (*18*), and (*19*) were more pronounced after treatments which promoted aggregation, such as dialysis, e.g., see Fig. 2.

A second electrophoresis of isolated electrophoretic zones (see Methods) demonstrated that the resolution of zones was reproduced when fractions of the whole pattern were analyzed (Fig. 1). Although migration of all zones was

*Zones unresolved under the stated conditions are indicated (*17, 18*) for example. Zones recognized since adoption of this convention are indicated by letters (*7a*), etc.

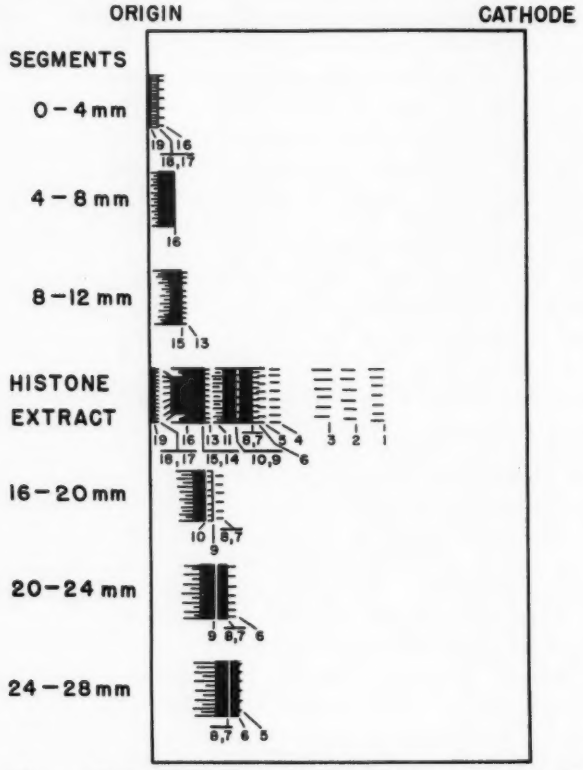

FIG. 1. Diagram illustrating the results of a second SGE of electrophoretic fractions of calf thymus histone. The top three and bottom three patterns were obtained from the segments indicated (in mm measured from the origin). The middle sample was whole histone extract. SGE was performed in a single wide gel at pH 5.1.

decreased because of the lower protein concentrations, the 'isolated' zones retained their expected relative mobilities and patterns of resolution. Because of non-planar fronts, especially in the vertical plane, overlapping of zones was not surprising. This evidence was consistent with the resolution of distinct components of the protein mixture.

The major regions of histone zones were identified with chromatographic fractions (see Methods). As Fig. 2 illustrates, peak sL (2) yielded zones (*13*) to (*16*), and some (*17*, *18*), while peaks sA_1 and sA_3 were more complex, the former consisting mainly of (*6*) to (*10*), the latter of (*8*) to (*10*). Both samples gave four zones in the region of (*17*, *18*), believed to represent aggregates arising during dialysis.

Since formation of histone aggregates is prevented by hydrogen-bonding agents (8, 9), the addition of 4 *M* urea to the gel–buffer system permitted electrophoresis at higher pH values (Fig. 4). When histone was subjected to SGE in 4 *M* urea in acetate buffer, pH 5.3, the pattern was similar to that in

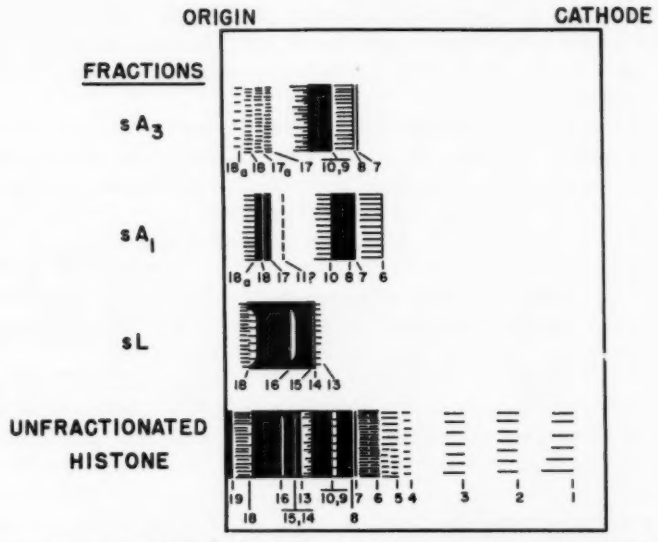

FIG. 2. Diagram of the results of SGE of chromatographic fractions of calf thymus histone. sL was lysine-rich histone (10); sA_1 and sA_3 were arginine-rich histones (all dialyzed). The bottom pattern was the original histone extract (undialyzed). SGE was performed in a single wide gel at pH 5.3.

the same buffer without urea at pH 4.9, but the mobilities were less. Hydrogen-bonded complexes did not contribute to the pattern of zones below pH 5. As pH was increased from 4.4 to 8.7, over-all mobilities decreased gradually but irregularly with greater differences in mobility and resolution accompanying certain pH changes. For example, from pH 6.3 to pH 7.2 the zones in the group (*6*) to (*10*) were clearly resolved, a new zone (*7a*) appeared at pH 6.3 and zones (*7b*) and (*9a*) at pH 7.2. Even at pH 8.7, as the isoelectric points of some components were approached (10), 11 zones could be distinguished. If all conditions and even trace components are considered, 22 zones have been observed. When histone was analyzed in starch gels in 7 *M* urea at several pH values, the patterns were the same as those illustrated in Fig. 4.

At times the best resolution was obtained in virtually unbuffered gels, presumably in which local pH depended largely upon the influence of the protein. For example a gel was made up in 0.004 *M* sodium barbiturate, 0.016 *M* barbituric acid, 0.016 *M* sodium chloride. The calculated pH of this weak buffer was 7.5, but the measured pH of the gel was 4.6. SGE clearly resolved 15 components from zones (*3*) to (*16*) (Fig. 5). The arc in zone (*16*) may have been due to local pH changes resulting in different mobilities across the zone.

An alternative method of preparation avoided the isolation of the nuclei from the gland before extraction of the histone (see Methods). The resulting protein solution was analyzed by SGE at several pH values in 4 *M* urea, in parallel with histone extracted from isolated nuclei from the same gland.

Figure 5 includes the patterns obtained at pH 5.9, 6.8, and 7.2 from the acid extract of whole tissue. These patterns do not differ from those from isolated nuclei at pH 6.3, 6.8, and 7.2 (Fig. 4) beyond the usual minor variations between similar preparations.

The stability of histone prepared from frozen thymus was compared with that from fresh tissue. The staining pattern (Fig. 6) was similar except that zones (*6*) and (*4*) had significantly greater intensities. After storage for 1 week at 3° histone from the frozen tissue had changed markedly. The staining intensity of zone (*6*) had increased greatly at the expense of zones (*7*) to (*11*). In contrast, histone extracted from fresh thymus showed no changes after 2 weeks of storage at 3°, and even after 3 weeks, despite perceptible microbiological contamination, changes in the patterns were less than those observed with frozen thymus.

The relative ultraviolet absorptions of the zone groups were obtained by scanning gels containing concentrated urea in order to gain additional evidence of the types of histones. While all histones are weakly absorbing, arginine-rich histones contain more tyrosine and phenylalanine, and have a greater optical density at 270 mμ than lysine-rich histones. Comparison of relative absorptions and staining intensities of the histone from frozen thymus (Fig. 6) indicated that group (*14*) to (*16*) was lysine-rich and group (*6*) to (*11*) was arginine-rich. The combination of substantial absorption with negligible staining near the origin suggests the presence of small amounts of non-histone material relatively rich in aromatic amino acids.

Determinations of N-*Terminal Amino Acids*

Because conflicting reports (4, 8) of the nature and proportions of the N-terminal residues have been attributed to different reaction conditions (6), determinations were made under several conditions as indicated in Methods. The results of these determinations are shown in Table I. Comparable propor-

TABLE I

N-Terminal amino acids of calf thymus nuclei and histone
(Yields in μmoles per 1.00 g histone)

					Histone II		Nuclei III	Histone III
Preparation	Histone I	Histone II		Nuclei II	Fresh	Stored	Frozen thymus	Stored
Reaction*	I	II		I	II	II	I	II
Hydrolysis†	I	I	II	I	Maxima of I and II	I	I	I
Ala	9.2	15.1	10.1	21.4	15.1	8.5	16.6	8.8
Pro	7.5	9.1	Trace	2.9	9.1	4.0	7.3	3.9
Val	0.2	0.8	2.6	2.0	2.8	15.9	1.4	7.9
V-P§	2.0	2.0	0.2	1.8				
Leu	—	—	Trace	4.3	Trace		4.9	4.8
Glu/Asp	—	1.2	0.5	6.7	1.2	5.1	3.1	13.4
Lys	Trace	0.5	0.3	1.1	0.5	1.1	2.7	0.9
Thr	—	0.5	0.5	0.2	0.5	0.3	1.7	0.6
Gly	0.8	0.9	Trace	4.6	0.9	0.5	3.4	—
Ser	0.6	—	Trace	—	Trace	0.8	2.0	—
Total	20.3	30.1	14.2	45.0	30.1	36.2	43.1	40.3

*Reaction: I, in two volumes of methanol; II, in saturated guanidine hydrochloride.
†Hydrolysis: I, in 11 *M* hydrochloric acid in sealed bulb for 4.5 hours; II, in 5.5 *M* hydrochloric acid with refluxing for 24 hours.
§V-P: valyl peptide.

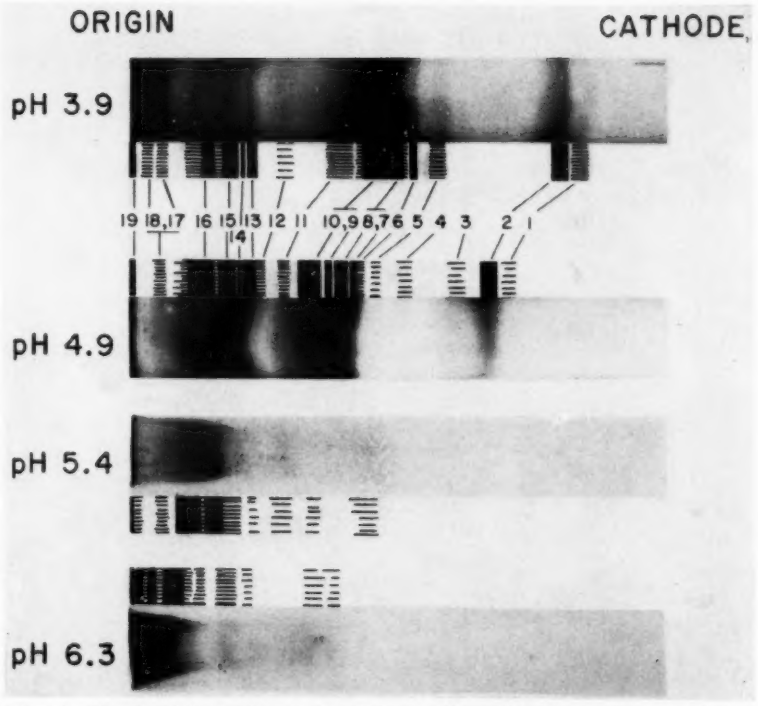

FIG. 3. Starch gel electrophoresis of calf thymus histone in absence of urea. pH values of the gels are indicated (μ 0.02, and length of runs, 6 hours at 3 v/cm, in all figures).

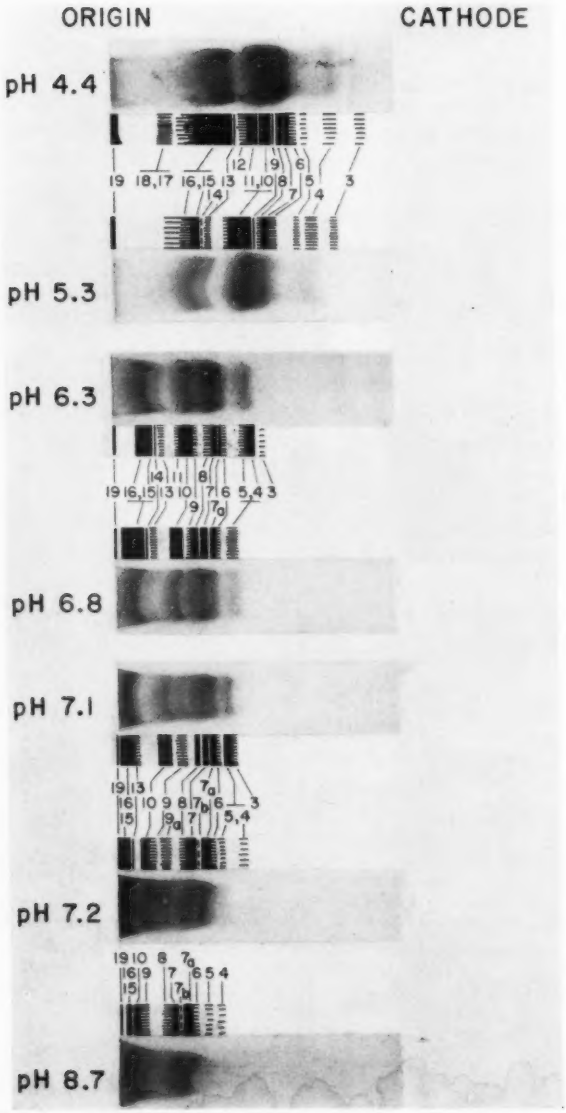

FIG. 4. SGE of calf thymus histone in gels containing urea. All samples were from the same extract; pH values are indicated.

PLATE III

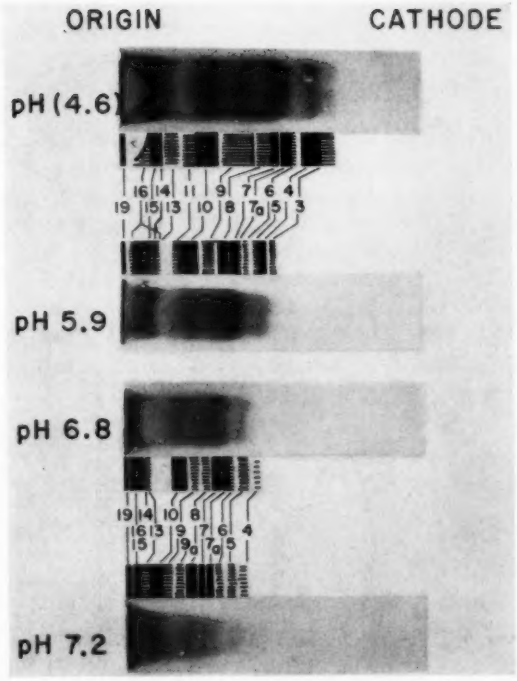

FIG. 5. SGE in gels containing urea. Upper pattern: histone from washed nuclei (unbuffered gel). Lower three patterns: histone from whole tissue (the nuclear preparations used for the experiments illustrated in Fig. 4 were isolated from the same gland).

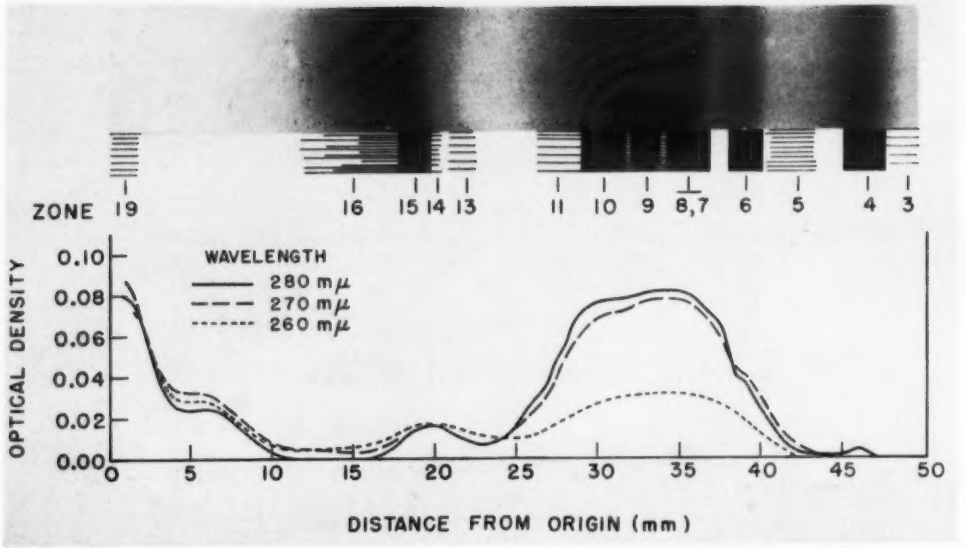

FIG. 6. SGE of histone from frozen calf thymus. Top: gel stained with amido black. Bottom: ultraviolet scanning of unstained gel; optical density readings made at the wavelengths indicated at 0.5-mm intervals measured from the electrophoretic origin.

tions of DNP-amino acids were found for two different protein preparations by the two methods of reaction with FDNB, although the better total yield was observed for the reaction in the presence of guanidine hydrochloride. The yield, about 30 μmoles from 1 gm of histone, was consistent with an average molecular weight of ca. 32,000, comparable to that reported by Phillips (4). The major N-terminal residues, alanine and proline, totalling 80% of the yield, also confirmed Phillips' findings. An additional 10% was valine remaining after sealed bulb hydrolysis mostly as a DNP-peptide near the origin of the paper chromatograms. The other amino acids of this peptide were not determined, but Luck *et al.* (8) found N-terminal valyl lysine in this protein.

Since end-group determinations should reveal any significant degradation during extraction of histone from nuclei, analyses of whole washed nuclei are included in Table I, along with determinations upon histone, which has undergone apparent degradation with prolonged storage. Comparison of the nuclei and histone of preparation II revealed that hydrolysis in the presence of deoxyribonucleic acid increased loss of DNP-proline and splitting of the valyl peptide. The DNP-glycine may have resulted from degradation of adenine (6). While extracted histone contained N-terminal alanine, proline, and valine only, the washed nuclei included proteins with N-terminal alanine, proline, valine, leucine, glutamic acid, and/or aspartic acid. With two different preparations the degradation apparent after storage led to decreases in yields of DNP-alanine and DNP-proline, great increases in DNP-valine, DNP-leucine, DNP-glutamic acid, and/or aspartic acid, and a slight increase in N-terminal lysine. These degraded histones showed marked changes in the electrophoretic patterns (already described), particularly in histone III, isolated from frozen thymus, which was particularly unstable and high in N-terminal glutamic acid.

Discussion

It has been possible to resolve a total of 22 zones from calf thymus histone under different conditions of starch gel electrophoresis. Of these, 16 appear to be reproducible native components. This degree of complexity of calf thymus histone is in agreement with a similar study on chicken erythrocyte histone. Earlier work using chemical separations (11), ultracentrifugation (10, 12), moving boundary (10, 12, 13), and zone electrophoresis in starch (probably not as a gel) (14), and cation-exchange chromatography (2, 15, 16) have demonstrated between two and five components of calf thymus histone with implications of further heterogeneity (6, 17).

The two main fractions of histone obtained by chromatography on Amberlite IRC-50, and by other methods, have been shown to be relatively rich in lysine and in arginine, respectively (18). These two fractions were identified with the two main groups of electrophoretic zones by SGE of chromatographic fractions and by determination of the relative optical densities of the zones at ultraviolet wavelengths. These two groups included most of the 19 zones resolved below pH 5 from calf thymus histone in gels containing buffers of low ionic strength.

Above pH 5, aggregation interfered with migration and resolution, unless the gels contained concentrated urea, which permitted SGE up to pH 8.7 without apparent aggregation. The use of unbuffered gels containing urea may assist resolution of the greatest number of zones but adds to the difficulty of reproducing conditions precisely. Of the 22 zones observed in various patterns at different pH values, the three fastest components occurred usually as traces and probably represented dialyzable contaminants of small molecular weight, while the three slowest were probably aggregated complexes partially dissociated at low pH.

Since patterns were essentially unchanged by urea below pH 5, complex formation dependent on hydrogen bonds did not contribute to the complexity. The use of such gels as a test for artifacts was recently mentioned by Connell and Smithies (19). Reproducible patterns have been obtained from many extracts from different thymus glands. The consistency of the separations was demonstrated by subjecting isolated zones to a second electrophoresis.

These histone preparations were relatively stable and compared well with those of other workers in sedimentation and chromatographic properties (2); however, in all reported preparations there has been little assurance that degradation had not occurred during the earliest steps. Since histone extracted from the tissue without prior isolation of nuclei was the same as that from isolated nuclei, degradation during nuclear isolation did not appear to contribute to the complexity. If significant degradation occurred during extraction with acid, the generation of new N-terminal amino acids would be expected, but no such new end-groups were found. Alanine and proline amounted to 80% of the total yield, that is, about one gram molecular weight per 30,000 g of histone; this was consistent with reports by Phillips (4, 17) and with recent physical measurements (9). The N-terminal valine confirmed the findings of Luck *et al.* (6, 8), but little lysine and leucine were found. The non-histone N-terminal residues of nuclei were probably contributed by residual chromosomal and nuclear membrane proteins.

Progressive degradation of histone during storage at 3° was not detected within 2 weeks, except in material from thymus that had been frozen. The release of proteolytic enzymes in frozen tissue is suggested by the increased amounts of N-terminal valine, leucine, and glutamic acid. The simultaneous decrease in recovered alanine and proline may result from the degradative production of small N-terminal peptides that could be lost in ether washings of DNP-protein. This degradation at pH 4 differed from that noted by Phillips and Johns at pH 7–9, which produced N-terminal alanine, lysine, and glycine (17).

The number of components resolved from histone by SGE is greater than that apparent with any other method. This sensitive technique could be applied with advantage to the comparison of histones from a variety of tissues and species.

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AMINO ACID PATTERN IN THE BLOOD OF RATS WITH DIETARY HEPATIC NECROSIS[1]

L. S. VALBERG[2] AND J. M. R. BEVERIDGE

Abstract

A paper chromatographic study of the amino acids and peptides in the blood of rats with dietary liver necrosis has been made.

The relative levels of alanine, methionine, tyrosine, glutamic acid, threonine, citrulline, glutamine, oxidized glutathione, and gamma aminoisobutyric acid were increased. The level of reduced glutathione was slightly reduced and levels of valine, taurine, and proline remained unchanged. Beta-alanine, which could not be detected in the blood of normal rats, was found in the case of those with acute liver damage.

Although arginine was present in the blood of normal animals and in those on a necrogenic diet prior to the development of the liver lesion, it was not detectable in those animals with acute hepatic necrosis. The possible implications of this finding have been discussed.

Introduction

Acute yellow atrophy in man has long been known to produce a generalized increase in the amino acid content of the blood and urine (1, 2). However, reports of the behavior of the individual amino acids in the blood of patients with liver disease, or in experimental animals with disorders of the liver, are limited and the results conflicting (3, 4, 5). To the best of our knowledge, no studies of the individual amino acids in the blood or plasma of experimental animals on diets designed to cause acute liver damage have been reported.

Methods and Materials

Five male weanling rats were depleted of their tissue reserves of vitamin E and selenium by being fed a diet in which the fat moiety was supplied in the form of vitamin-E-stripped corn oil and cod liver oil. The composition of the basal diet was as follows: alcohol-extracted baker's yeast, 40%; sucrose 43.6%; vitamin-E-stripped corn oil, 9%; cod liver oil, 1%; choline, 0.3%; salt mixture, 3%; vitamin mixture, 0.7% (6); celluflour, 2%; DL-methionine, 0.4%. The occurrence of liver necrosis was prevented by the high level of yeast and the inclusion of 0.4% methionine in the ration. After 50 days on this preparatory regimen, liver necrosis was induced by removing the supplement of methionine from the diet and lowering the level of yeast.

When it was obvious that liver necrosis had developed, the animals were anaesthetized by intraperitoneal injection of a solution of Nembutal at a dosage of 25.0 mg/kg body weight, and blood was obtained with a syringe from the abdominal aorta at its bifurcation. Powdered heparin was used as an anti-coagulant. As a control, blood was obtained from five normal rats which had

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[2]The work reported here was done in partial fulfilment for the requirements of the M.Sc. degree in Biochemistry and was performed during the tenure of a Canadian Life Insurance Medical Fellowship.

been fasted for a period of 24 hours, a period that corresponded approximately to the period of anorexia in the animals that developed liver necrosis. The amino acid nitrogen content of the blood was determined by the method of Frame and Russell (7).

Immediately after collection, 1 ml of blood was added to 1 ml of 0.2 *M* N-ethylmaleimide (8) in a 50-ml round bottom glass-stoppered centrifuge tube. Deproteinization of the blood was effected with picric acid as described by Hamilton and Van Slyke (9). The excess picric acid was removed by passing the supernatant through a column of Dowex 1 (10). The effluent was evaporated to dryness on a rotary evaporator and the residue was reconstituted in 0.3 ml of 0.1 *N* HCl.

Before adopting picric acid for the deproteinization of blood, an attempt was made to apply the method suggested by Smith and Tuller (11). This procedure, which involves the coagulation of protein with heat, invariably produced a filtrate which was contaminated with hematin, a circumstance that interfered with the subsequent chromatography of the amino acids. Smith and Tuller also advocated the use of a cation exchange resin to obtain the amino acids free from inorganic ions and hematin. Although this step eliminated the hematin, it was found that the ammoniacal solution required to elute the amino acids from the resin produced marked degradation of the sulphur-containing amino acids and peptides, and the N-ethylmaleimide derivatives.

The amino acids were separated into two groups by descending one-dimensional chromatography on Whatman No. 3MM paper which had previously been washed with solvent. The protein-free filtrate was transferred by means of a micro-pipette to a pencilled line 3 in. from the top of a paper strip 1 in. × 22 in. After a short preliminary period of equilibration in the presence of the solvent, the chromatogram was developed in tertiary butanol, methyl ethyl ketone, water, and pyridine (50:50:20:25). After 26 to 28 hours the paper was removed from the solvent, dried, and traces of solvent were removed by suspending the strips in a specially constructed steam chamber (Fig. 1) at a temperature of 74–76° C for 8–10 minutes (12). A narrow strip was cut from the center of the chromatogram and the amino acids detected with ninhydrin. On the basis of the result, the chromatogram was divided into a proximal portion containing proline and the amino acids that moved more slowly than proline (fraction No. 1), and a distal portion containing amino acids that moved more rapidly (fraction No. 2). The amino acids were eluted from the paper by means of descending chromatography with water:ethanol:formic acid (40:30:30). The proximal strip was eluted in the opposite direction to which it was run initially to ensure complete removal of the slower-moving amino acids. The eluates were evaporated to dryness on a rotary evaporator and the residue was taken up in 0.02 *N* HCl. In order to remove any traces of picric acid that remained, the solution was passed through a column of Dowex 1, 1 cm × 1 cm, followed by three washings of 0.02 *N* HCl. The effluent was evaporated to dryness on a rotary evaporator and the residue was reconstituted in 0.1 ml of 0.1 *N* HCl in 10% isopropanol. Amino acid nitrogen determinations were performed on 0.02-ml aliquots.

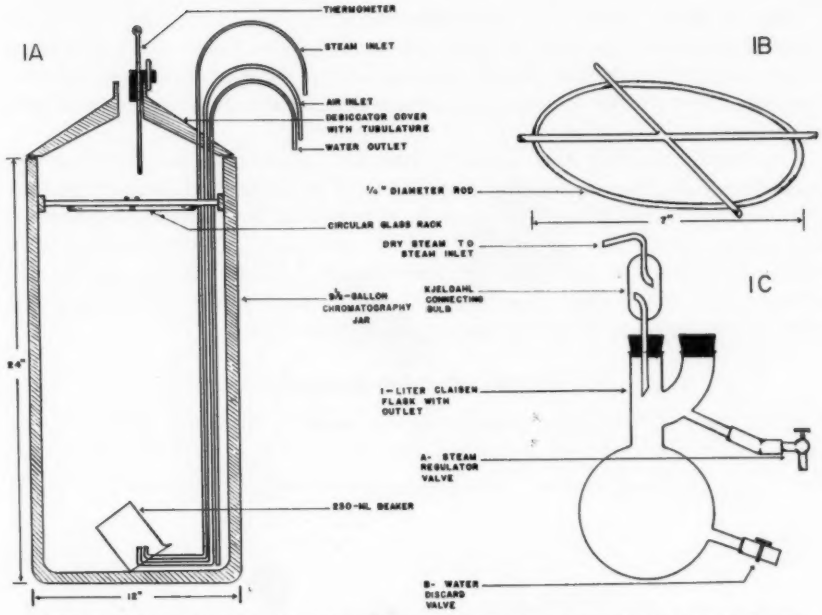

FIG. 1. Steam chamber: 1A, schematic drawing of chromatography jar and desiccator top used for steam chamber; 1B, design of circular rack; 1C, apparatus for cleaning steam.

The separation of the amino acids in fraction No. 1 and No. 2 was accomplished by two-dimensional chromatography. Samples of eluates containing 0.02, 0.04, 0.06, and 0.08 mg of amino nitrogen were applied to sheets of Whatman No. 3MM paper, 18 in. × 22 in. Because of the instability in alkaline media of compounds conjugated with N-ethylmaleimide and compounds with disulphide linkages, the solvent systems were made slightly acid. The amino acids were separated in the first direction by developing the chromatogram in Mallinckrodt liquified phenol:water (90:10), which was run in an ascending manner up the shorter side of the paper. Tailing of the spots which frequently occurs when phenol is used as a solvent was minimized by incubating the chromatograms in the chamber with both phenol and water vapor for 10 to 12 hours, the chamber being saturated with steam just prior to the equilibration period. Sodium cyanide, commonly used in chromatography to retard the decomposition of phenol was not used for it was found to cause reduction of cystine, glutathione, and the N-ethylmaleimide derivatives. When phenol had travelled to within 1 in. of the top edge of the paper, the chromatogram was dried at 40° C for 4 to 6 hours and phenol that remained on the paper was removed in the steam chamber.

The chromatogram that contained fraction No. 1 was then run in the second solvent, hexyl alcohol, methyl ethyl ketone, water, and acetic acid (50:50:20:25) in a descending manner. It was removed from the solvent after 45 to 48 hours and dried.

The chromatogram that contained fraction No. 2 was developed in a descending manner in tertiary butanol, amyl alcohol, methyl ethyl ketone, water, and pyridine (25:35:40:20:12.5) for 40 hours. The chromatogram was then dried and the last traces of pyridine removed from the paper in the steam chamber.

The chromatograms were sprayed with 0.5% ninhydrin in ethanol as described by Kay, Harris, and Entenman (13). The sulphydryl compounds that were conjugated with N-ethylmaleimide were located by a specific reaction for this group (14). The paper was sprayed with 0.6% N-ethylmaleimide in isopropanol, dried in the ventilating oven at 40° C for 10 minutes, and finally sprayed with 2% KOH in isopropanol. Sulphydryl compounds conjugated with N-ethylmaleimide showed up as reddish-pink areas.

Results and Discussions

When the supplementary methionine was removed from the ration and the yeast content lowered from 40 to 18%, the animals died of liver necrosis within

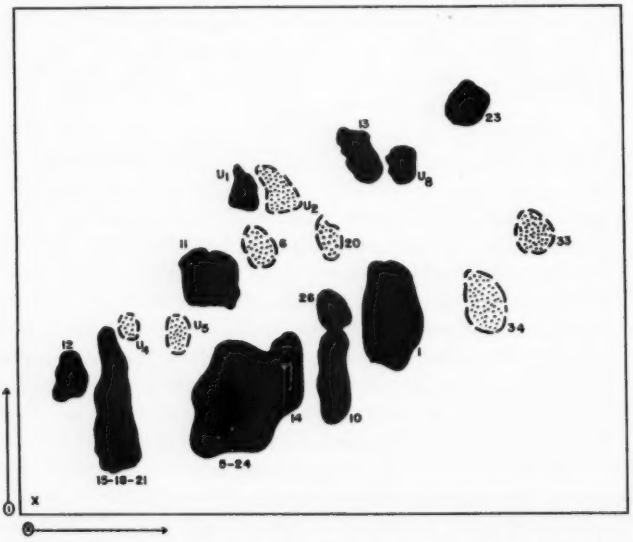

FIG. 2. A two-dimensional chromatogram of fraction No. 1 obtained from blood of rats with liver necrosis. Amounts equivalent to 0.19 ml of blood were applied to the paper (0.04 mg amino acid nitrogen). The solvent systems were: (*a*) phenol:water (90:10); (*b*) hexyl alcohol:methyl ethyl ketone:water:acetic acid (50:50:20:25). Note that arginine (3) is absent.

1. Alanine
5. Aspartic acid
6. Citrulline
10. Glutamic acid
11. Glutamine
12. Oxidized glutathione
13. Glutathione N-ethylmaleimide
14. Glycine
15. Histidine
18. Lysine
20. Methionine sulphone
21. ?Ornithine
23. Proline
24. Serine
26. Threonine
33. Gamma-aminoisobutyric acid
34. Beta-alanine

U_1. Unidentified
U_2. Unidentified
U_4. Unidentified
U_5. Unidentified
U_8. Unidentified

60 hours. The amino acid nitrogen content of the pooled sample of "necrotic" blood was 25.2 mg/100 ml, compared to 8.4 mg/100 ml for the control group.

The amino acid patterns on two-dimensional chromatograms of rats with liver necrosis are shown in Figs. 2 and 3. A semi-quantitative comparison of

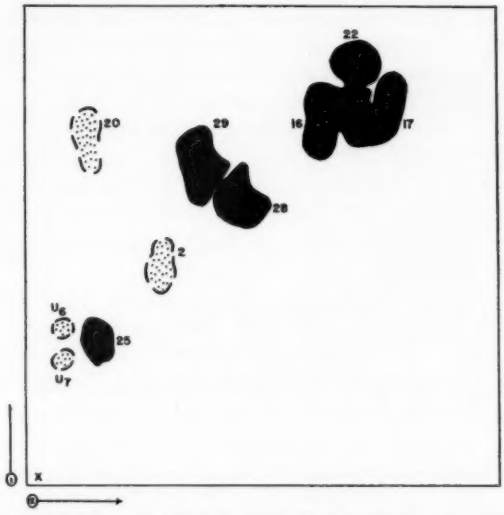

FIG. 3. A two-dimensional chromatogram showing separation of fraction No. 2 obtained from the blood of rats with liver necrosis. Amounts equivalent to 0.77 ml of blood were applied to the paper. The solvent systems were: (*a*) phenol:water (90:10), (*b*) tertiary butanol:amyl alcohol:methyl ethyl ketone:water:pyridine (25:35:40:20:12.5).

2. Alpha-aminoisobutyric acid
16. Isoleucine
17. Leucine
20. Methionine sulphone
22. Phenylalanine
25. Taurine
28. Tyrosine
29. Valine
U_6. Unidentified
U_7. Unidentified

the amino acids of blood of normal rats and animals with liver damage is shown in Table I; other unidentified ninhydrin reactive substances are shown in Table II. It should be noted that the comparison of the amino acids is based on the results obtained from four aliquots of normal and "necrotic" blood containing similar amounts of amino acid nitrogen (0.02, 0.04, 0.06, and 0.08 mg) and not on the basis of similar volumes of blood. If the results are compared on the latter basis there is an increase in the absolute concentration of all the amino acids that were detected with the exception of arginine.

The relative concentrations of alanine, glutamine, methionine (in the form of methionine sulphone), and tyrosine were increased, whereas the level of reduced glutathione was slightly reduced in the blood of rats with liver necrosis. The groups of amino acids represented by aspartic acid, serine, and glycine, and by glutamic acid and threonine were increased, whereas the leucine, isoleucine, phenylalanine group appeared to be slightly decreased.

The precise reason for the increase in the blood levels of alanine, methionine, and tyrosine and the groups of amino acids represented by aspartic acid, serine,

TABLE I

Semiquantitative determination of amino acids of blood of normal rats and animals with liver necrosis*
(++++ very dense; +++ dense; ++ medium intensity; + small intensity; ? presence doubtful)

	Normal blood	Necrotic blood
Alanine	+++	++++
Arginine	++	Absent
Aspartic acid, serine, glycine	++	+++
Alpha-aminoisobutyric acid	+	+
Beta-alanine	?	+
Citrulline	+	+
Glutamic acid, threonine	++	+++
Histidine, ornithine (?), lysine	+++	+++
Glutamine	+	++
Leucine, isoleucine, phenylalanine	++	+
Methionine sulphone	+	++
Proline	+	+
Oxidized glutathione	Absent	+
Reduced glutathione	+++	++
Gamma-aminoisobutyric acid	Absent	+
Taurine	+	+
Tyrosine	+	++
Valine	+	+

*Based on the results obtained from aliquots containing the same amount of amino acid nitrogen.

TABLE II

Unidentified ninhydrin-reactive substances in blood of normal rats and animals with liver necrosis*
(++++ very dense; +++ dense; ++ medium intensity; + small intensity; ? presence doubtful)

Identification	Normal blood	Necrotic blood
U_1 (Fig. 2)	+	+
U_2 (Fig. 2)	+	+
U_3 (above and to the left of U_1)	+	Absent
U_4 (Fig. 2)	?	+
U_5 (Fig. 2) ? asparagine	Absent	?
U_6 (Fig. 3)	?	?
U_7 (Fig. 3)	?	?
U_8 (Fig. 2)	Absent	++

*Based on the results obtained from aliquots containing the same amount of amino acid nitrogen.

and glycine, and of glutamic acid and threonine is not known. There are at least three possible sources of the amino acids: necrotic liver tissue; release of free amino acids from tissue cells where they are present in 5 to 10 times the concentration of blood (15); breakdown of tissue protein. Although the synthesis of protein from amino acids and the breakdown of protein are in balance in the normal adult animal, the equilibrium for this reaction is far in the direction of degradation and energy must be expended to prevent the breakdown of tissue protein. In animals with liver necrosis there is not only a disturbance in energy-yielding mechanisms in the liver but also profound hypoglycemia (16, 17) which deprives other tissues of an important source of energy. That hypoglycemia might be related to the increase of amino acids in the blood is suggested by the work of Flock and co-workers (18), who observed that

administration of glucose to hepatectomized animals minimized the increase in the level of amino acid nitrogen.

The finding of beta-alanine, which is not a constituent of tissue protein, in the blood of animals with liver necrosis is interesting. Presumably, it has been liberated from such compounds as coenzyme A, anserine, and carnosine.

Oxidized glutathione was present in the blood of animals with liver damage but it was not detected in normal blood. This finding is consistent with that of Lindan and Work (19), who reported a decline of reduced glutathione in the blood and a marked decrease in the ratio of reduced to oxidized glutathione in the livers of animals with hepatic necrosis.

Cysteine was not detected in normal or "necrotic" blood with either ninhydrin or reagents that specifically identify conjugated maleimide derivatives. Cystine formed a very diffuse spot below and to the left of oxidized glutathione and was found in the blood of both normal rats and in animals with liver damage.

The most noteworthy finding was the complete absence of arginine from the blood of animals with liver necrosis. This was confirmed by spraying several of the chromatograms with alpha naphthol:hypochlorite solution, which gives a red color with arginine. In addition, samples of blood, obtained from rats on necrogenic diets prior to and at the time of development of liver necrosis, were specifically tested for arginine. Twenty male weanling rats were fed a basal diet essentially free from fat. After 100 days on this regimen the rats were divided into two groups: group A remained on the basal diet while group B was fed the basal ration containing 50% lard, a modification designed to induce acute liver damage within 24 or 48 hours. Rats from each group were selected at 3- to 6-hour intervals and anaesthetized, and a sample of blood was obtained from the abdominal aorta. The blood was pooled in three samples: A, obtained from rats in control group A; B_1, from animals in group B that were on the necrogenic diet but had livers which were histologically normal at the time of sacrifice; and B_2 from animals with massive liver necrosis. A sample of blood from normal rats on chow diet was also subjected to analysis. The samples were deproteinized with picric acid, and aliquots of the filtrate, corresponding to 3 ml of blood, were chromatographed. After development, the arginine was detected by spraying the chromatograms with alpha naphthol:hypochlorite reagent. The results are shown in Fig. 4. It will be seen that arginine was present in the blood of normal animals, and in those receiving a necrogenic diet (group B_1, prenecrotic), but that none or at most only a trace of arginine was present in the rats that had developed acute liver necrosis (group B_2, necrotic).

The cause and significance of the marked fall in arginine is not definitely known but one or two probabilities may be mentioned. Since the enzyme arginase is espcieally abundant in liver tissue, it is possible that the fall in arginine in the peripheral blood is due to the liberation of arginase from dead and dying liver cells. Of possible pertinence in this regard is the report by Manning and Grisolia (20) that serum arginase activity is, on the average, higher in patients with liver disease than in normal subjects. A depression of arginine levels has not been reported in patients with liver disease. In a study

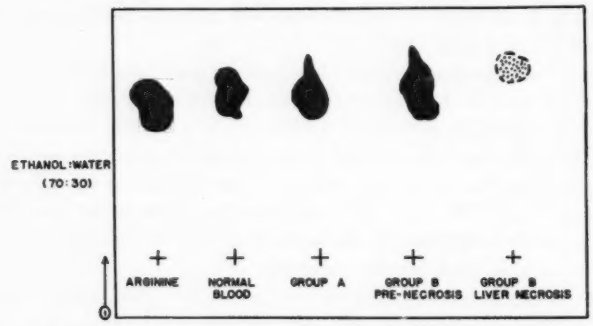

FIG. 4. A one-dimensional chromatogram stained specifically for arginine.

of the amino acid pattern of the plasma of patients with acute hepatic failure Walshe (3) and Iber *et al.* (5) reported an absolute increase in the level of arginine whereas Pearl and McDermott (21) found no change in arginine levels in the blood of their patients.

Thus, quite apart from the disruption of metabolism in the necrotic liver, the absence of arginine imposes an additional limitation to the detoxification of ammonia and to the synthesis of urea. It has been shown in experimental animals that high doses of ammonia lead to coma, convulsions, and death (22). Implication of arginine as a protective factor against ammonia toxicity in the rat was demonstrated by Greenstein and collaborators (23, 24, 25). They showed that the administration of L-arginine 60 to 90 minutes prior to the administration of a lethal dose of ammonium acetate completely protected the animals. The authors suggested that the protective effect of arginine may be mediated by the urea cycle. The absence of arginine in the blood of a rat with massive liver damage presumably would render it extremely susceptible to the toxic effects of ammonia. The accumulation of ammonia in the blood (26) and tissues might contribute to the rapidity with which death occurs when liver necrosis develops.

Citrulline was slightly increased in the blood of animals with liver damage. Elevations of citrulline in the blood of patients with increased levels of ammonia and cirrhosis of the liver have been reported by Pearl and McDermott (21). These workers assumed that since citrulline is not ordinarily a component of dietary protein and is synthesized from ornithine, the amount present in the peripheral blood was a direct reflection of citrulline metabolism in the liver. On this basis they postulated that in liver cirrhosis the conversion of citrulline to arginine might be a rate-limiting step in the synthesis of urea. In the present study it is probable that the elevation of citrulline was due at least in part to a defect in the formation of arginine and to the liberation of citrulline from the liver cells.

Several unidentified ninhydrin reactive substances were detected (Table II). In the blood of normal rats there were traces of three unidentified substances (U_1, U_2, U_3) above citrulline and traces of two adjacent to taurine. The unidentified substance U_4 (Fig. 2) increased in concentration in the "necrotic"

blood; whereas U_3, present in normal blood, disappeared. The substance U_5 (Fig. 2) gave an orange-brown color with ninhydrin, and it was believed to be asparagine. There was no evidence of U_8 (Fig. 2) in normal blood. The identification of these substances remains a problem for future investigations.

Acknowledgments

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BLOCKADE OF SOME CARDIAC ACTIONS OF ADRENALINE BY DICHLOROISOPROTERENOL[1]

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With the technical assistance of SYLVIA NEGRYCH

Abstract

Dichloroisoproterenol (DCI) has been found to produce a competitive blockade of the positive inotropic action of adrenaline on cat papillary muscles. The effect of adrenaline on electrical excitability of this preparation also is blocked. DCI itself has been shown to have considerable adrenaline-like activity, increasing the strength of contraction and decreasing the threshold of electrical excitability. The positive chronotropic and arrhythmia-inducing effects of adrenaline have been shown to be blocked in the whole animal.

There has been considerable interest during recent years in the effects of dichloroisoproterenol (DCI) a compound first reported by Powell and Slater (1) to block the peripheral inhibitory and cardiac actions of adrenaline. Moran and Perkins (2) have described the blockade by this compound of some cardiac effects of adrenaline in dogs and rabbits. Several other studies, not yet published in detail, have been summarized by Furchgott (3). We have studied this drug and its effect on the action of adrenaline in the isolated cat papillary muscle because this preparation readily demonstrates several cardiac actions of adrenaline and served to reveal the mechanism of the blockade induced by DCI. It became apparent early in the course of this work that the blocking agent had considerable intrinsic activity which had not been observed in some of the earlier studies, and this report also considers this aspect of the pharmacology of dichloroisoproterenol.

Methods

Papillary muscles were obtained from the right ventricle of cats killed by a blow on the head. They were suspended immediately in a medium containing NaCl 6.0, KCl 0.354, $CaCl_2.2H_2O$ 0.350, KH_2PO_4 0.081, $MgSO_4.7H_2O$ 0.147, $NaHCO_3$ 2.1, and glucose 0.9 g/liter, which was oxygenated and buffered to a final pH of 7.3–7.4 by a rapid flow of 5% CO_2–95% O_2. It was found that in this medium small concentrations of adrenaline induced automaticity which interfered with evaluation of inotropic effects. In order to obtain adequate dose–response curves it was necessary to increase the potassium concentration of the medium from 5.35 meq/liter to 7.73 meq/liter by increasing the KCl concentration to 0.531 g/liter. The muscles were stimulated by 10 millisecond rectangular pulses delivered at a rate of 60/minute through silver electrodes placed near their base. The threshold of electrical excitability was determined by stepwise increases of the stimulating voltage until three successive stimuli were followed by contractions (4). Papillary muscles react to electrical stimula-

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Contribution from the Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Man. The work was aided by grants from the National Research Council of Canada and Eli Lilly Co.

tion in an all-or-none manner. The force of contraction was recorded quasi-isometrically using a brass lever arm to which Baldwin strain gauges had been cemented. Movement was always less than 1.5 mm. The strain gauges formed two arms of a Wheatstone bridge; the current flow was recorded by a Grass polygraph. Since each muscle served as its own control, strengths of contraction were compared on the basis of millimeter of pen excursion at constant amplification.

Duplicate cumulative adrenaline dose–response curves were obtained prior to blockade. Each curve was determined using fourfold increments in concentration selected so that the composite curves contained points separated by twofold concentration differences. After exposure of the muscle to DCI, a single curve with twofold concentration increments was determined. All results were expressed in per cent of the maximal response of the muscle as determined in the control runs.

Solutions of adrenaline bitartrate and dichloroisoproterenol hydrochloride were made fresh daily in the same medium as was used in the tissue bath. Adrenaline solutions were acidified; their concentrations were expressed in terms of the base. The volumes added to the bath did not exceed 3 ml even during the repeated additions required to obtain the dose–response curves. The muscles were exposed to the blocking agent for a period of 10 minutes, after which the fluid in the bath was changed several times. The new series of measurements were made 10–20 minutes after washing, at which time the muscles appeared to have reached a new steady state. Each muscle was tested at only one concentration of DCI.

Dogs anesthetized with 30 mg/kg of pentobarbital sodium were used to test the antiarrhythmia effect of DCI in vivo. In most experiments, the dose of adrenaline necessary to induce arrhythmia was 16 μg/kg injected intravenously over a 15-second period. Arrhythmia was defined as a minimum of three successive ventricular electrical systoles having predominantly downward QRS complexes not preceded by P-waves. After two control injections at a 30-minute interval, a small dose of DCI was injected. Fifteen minutes after DCI, the dose of adrenaline was repeated, followed if protection was absent or incomplete by the next higher dose of DCI. This procedure was repeated until complete protection (absence of abnormal electrical complexes) was obtained. Since DCI is a long-acting drug, the protecting dose was expressed as the cumulative dose injected.

Results

1. Action of Dichloroisoproterenol on Papillary Muscles

We were impressed immediately by the high intrinsic activity of DCI in our preparation (Fig. 1). The minimal inotropic response was obtained at a concentration of 2.5×10^{-8} g/liter, which could not be shown to have any significant blocking action under the conditions of our experiments. The inotropic action was well maintained in most muscles for several hours and repeated washings. Table I shows that DCI, like adrenaline, lowers the threshold for electrical stimulation. This effect was observed while the muscle was

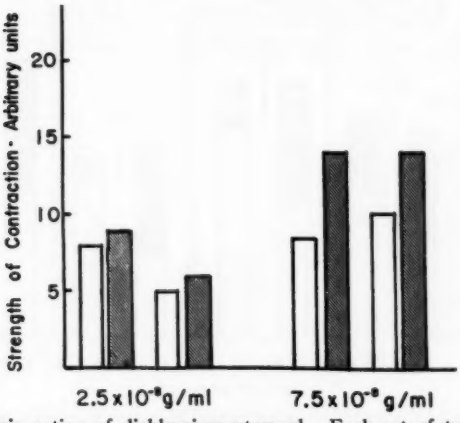

FIG. 1. Inotropic action of dichloroisoproterenol. Each set of two bars represents one cat papillary muscle preparation. The open bars show the strength of contraction before, the shaded bars that after addition of the indicated concentration of DCI.

TABLE I

The effect of dichloroisoproterenol on the threshold of electrical excitability

Concentration, g × 10^{-8}/ml	Number of muscles	Mean values of: Control threshold (volts)	Post drug threshold (volts)	% decrease
7.5	2	1.00	0.89	11.0
12.5	6	1.26	1.22	3.2
25	7	0.74	0.67*	9.5
50	1	0.80	0.73	8.8

*Significant ($P < .05$) by analysis of paired data.

exposed to the blocking agent. In most muscles, the threshold rose during the period following removal of the agent from the bath until a new steady state, with a threshold higher than control, was reached. In three muscles which were beating spontaneously at rates of 65–80/minute, DCI 2.5–25×10^{-8} g/liter increased the frequency of the contractions by 40–60%.

2. Effect of Dichloroisoproterenol on the Responses of Papillary Muscles to Adrenaline

A. Observations in Normal (5.35 meq/liter) Potassium Medium

Low concentrations of DCI were effective in decreasing the inotropic action of adrenaline. The results of four experiments using two concentrations of DCI are shown in Fig. 2. In the particular experiments illustrated, 1.25×10^{-7} DCI had a pronounced inotropic effect. The response to adrenaline subsequent to the blocking agent was decreased, but the maximum strength of contraction obtained was as great as in the control experiments. This allows the possibility that no real blockade was present. However, this was not true of all muscles used, and it is highly improbable that either of the responses illustrated represent the maximal possible strength since considerably stronger contractions were recorded in the experiments described below (Section 2-B). Higher

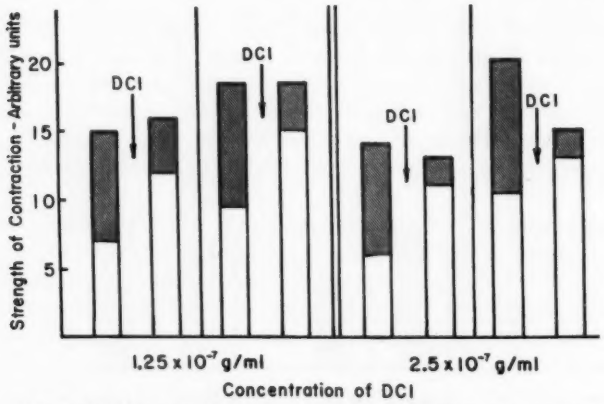

FIG. 2. Effect of DCI on the inotropic action of adrenaline on cat papillary muscles in a medium containing 5.35 meq/liter potassium. Each set of two bars represents one preparation. The open parts of the bars show the control strength of electrically stimulated contractions, the shaded portions the effect of adrenaline. Between determinations, DCI was added to the bath to yield the concentrations indicated.

concentrations of adrenaline could not be used in this series of experiments to test the maximal response because of the development of automaticity. There is no doubt concerning the blockade of the inotropic action of adrenaline by the higher concentration of DCI shown in Fig. 2. The minimal concentration of DCI at which blockade could be observed was 7.5×10^{-8} g/ml. We observed a 20% potentiation of the inotropic response to adrenaline in three of four muscles tested after exposure to 2.5×10^{-8} g/ml of DCI. This was, however, not statistically significant. We have no explanation for this potentiation, but it is comparable to a slight, also not statistically significant, potentiation of the vasodilator response to adrenaline which we have observed after very small doses of DCI (unpublished observations).

The decrease in the threshold to electrical stimulation induced by adrenaline also appeared to be blocked in three, and attenuated in four of seven muscles after treatment with DCI at concentrations of 1.25 and 2.5×10^{-7} g/ml. However, as has been pointed out above, the threshold of muscles treated with DCI and then washed was increased. This means that the control thresholds from which the adrenaline action was measured were higher than the control thresholds prior to DCI.

B. Observations in "High" (7.73 meq/liter) Potassium

In order to prevent the induction of automaticity by adrenaline and thereby make possible the construction of dose–response curves for its inotropic action over an adequate concentration range, the potassium content of the medium was increased. Under these conditions, the blocking potency of dichloroisoproterenol was increased by at least fivefold. A significant decrease in the inotropic action of adrenaline was obtained with 1.25×10^{-8} g/ml of DCI, the lowest concentration tested. The blockade of responses to four different concentrations of adrenaline by three different concentrations of DCI is shown

in Fig. 3. We have no explanation for the anomalous point corresponding to the adrenaline concentration of 1.28×10^{-6} g/ml after pretreatment with

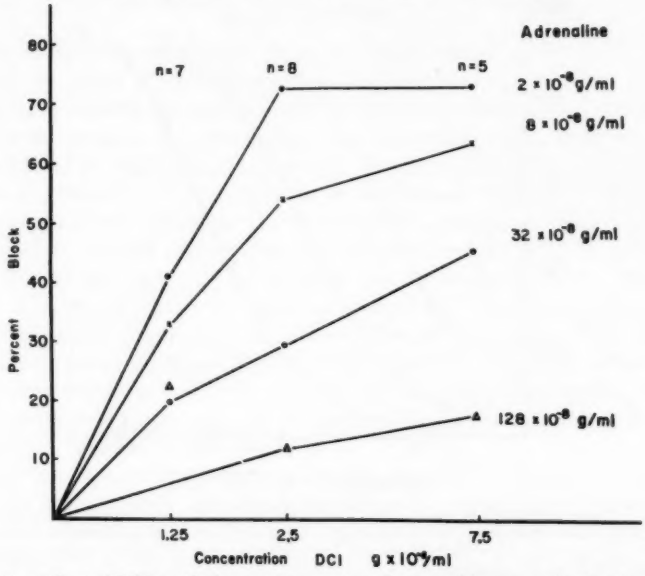

FIG. 3. Effect of DCI on the inotropic response of cat papillary muscles to adrenaline in a medium containing 7.73 meq/liter potassium. Blockade is expressed as per cent of the control response. "*n*" is the number of muscles for which the point represents the mean.

1.25×10^{-8} g/ml of the antagonist. Figure 4 shows two of the dose–response curves from which the data in Fig. 3 were plotted. The curves were parallel, but the maximal responses after blockade appeared to be smaller than the control maximal responses. However, induction of automaticity limited the range of concentrations of adrenaline which could be used in many of the experiments. In an attempt to check this point further, with its implication of non-equilibrium blockade, a few muscles were tested at an antagonist concentration (7.5×10^{-7} g/ml), 10 times greater than the highest concentration used in the initial study. This concentration decreased the inotropic effect of calcium ion, indicating some non-specific cardiac depressant action. Parallelism of the dose–response curves was maintained, and in some muscles the very high concentrations of adrenaline used allowed us to reach the same maximal responses as in experiments with lower concentrations of DCI, or even as in the control runs. The dose–response curve for these experiments is also plotted in Fig. 4. These additional data indicate strongly that blockade of the inotropic response of the cat papillary muscle to adrenaline by DCI is indeed a competitive antagonism.

3. Effect of Dichloroisoproterenol on Some Responses to Adrenaline in vivo

Swain (personal communication) has shown that DCI prevents ventricular fibrillation due to adrenaline in animals sensitized with methyl chloroform.

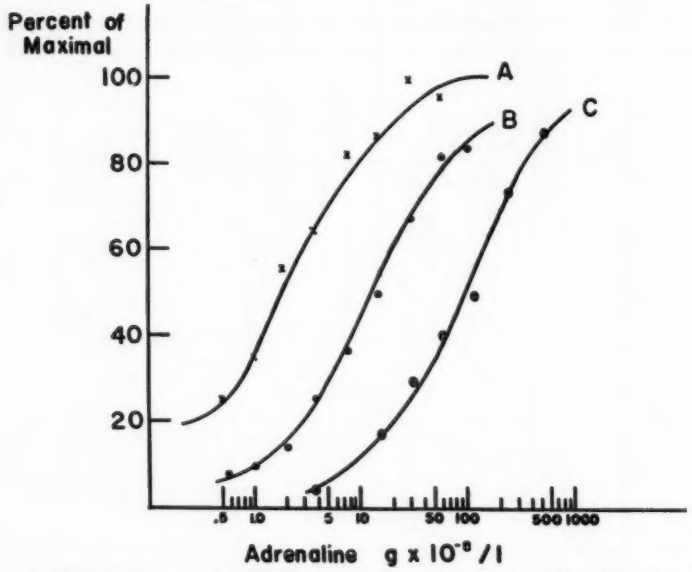

FIG. 4. Dose–response curves of the inotropic action of adrenaline in cat papillary muscles suspended in a medium containing 7.73 meq/liter of potassium. (A) control; (B) after exposure to 2.5×10^{-8} g/liter DCI for 10 minutes; (C) after exposure to 7.5×10^{-7} g/liter DCI for 10 minutes.

We have found DCI capable of preventing cardiac arrhythmias induced by adrenaline in non-sensitized dogs. Table II shows the results and indicates a

TABLE II

Protection by dichloroisoproterenol against adrenaline-induced cardiac arrhythmia

Dose of DCI μg/kg	6	12.5	25	50	100	500
No. dogs tested	2	5	3	10	8	3
Cumulative no. protected	0/2	2/5	2/5	7/12	10/13	13/13

very flat dose–response curve for DCI protection under the conditions of our experiments. In the compilation of this table, it has been assumed that a dog protected by a low dose of DCI would also have been protected by higher doses. The number of animals actually tested at a particular dose is shown in the appropriate column. The large dose of adrenaline used caused a transient severe tachycardia, which was changed to a bradycardia when the peak reflex vagal discharge occurred. Small doses of DCI (0.05 mg/kg) blocked this tachycardia. Potentiation of the vagal slowing was seen with doses greater than 0.05 mg/kg. This potentiation may be explained as a blockade of the chronotropic action of the injected adrenaline. Blockade of this action by DCI allows the vagal discharge to act on the SA-node unopposed. Doses greater than 0.1 mg/kg caused no greater augmentation of the vagal slowing but

increased the duration of the bradycardia. Heart rates were determined only during sinus rhythm.

Discussion

Dichloroisoproterenol appears to be a potent competitive blocking agent which will be useful in the elucidation of adrenergic mechanisms. However, two actions of this compound decrease its value for this purpose. These are its high intrinsic activity in some tissues, and the non-specific depression of the heart produced by high concentrations of the agent. The intrinsic activity is of sufficient magnitude in our preparation to be the only observable effect at very low concentrations. However, its importance appears to vary in different tissues. Thus, Powell and Slater (1) did not observe intrinsic activity on isolated guinea pig tracheal chains or on the cat uterus in vivo. Moran and Perkins (2) reported that only the first dose of DCI had a positive inotropic action, subsequent doses showing only cardiac depression. Since they used rather large, increasing doses of DCI, it is possible that a major effect of their repeated injections was a cumulative non-specific cardiac depression. We have observed some additional increase in heart rate on injection of a second small dose of DCI.

We have no explanation for several results obtained in this work. We do not know why an extremely low concentration of a competitive blocking agent should potentiate the action of the agonist. Others have observed potentiation of adrenergic responses by small doses of a variety of adrenergic blocking agents (5). There does not appear to be any clear explanation of the observation that the decrease in threshold for electrical stimulation due to DCI occurred only during exposure of the papillary muscle to the agent whereas its positive inotropic and adrenergic blocking actions were well maintained after washing. The increase in potency of DCI as a blocking agent when the medium was changed to one containing an increased concentration of potassium is of considerable interest. The reason for this change is not known. It is not accompanied by an increase in the inotropic action or cardiac depressant actions of DCI in the high potassium medium. In addition, a separate series of experiments in which the inotropic action of several concentrations of adrenaline was tested on the same muscles at the two concentrations of potassium showed no alteration in the response due to the different ionic composition of the medium.

Acknowledgments

I would like to thank Dr. I. H. Slater for generous supplies of dichloroisoproterenol. Mr. L. Hildebrand and Mr. S. Hershfield performed some of these experiments as part of their Clinical Science research projects.

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PAPER ELECTROPHORESIS OF SERA AND PLASMA FROM CHICKS AFFECTED WITH ERYTHROBLASTOSIS[1]

C. LE Q. DARCEL

With the technical assistance of H. C. KLASSEN

Abstract

Plasma and sera from normal and leukemic (erythroblastosis) chickens have been studied with the aid of paper electrophoresis. Differences are more easily demonstrable with pH 6.4 phosphate buffer than with barbiturate buffer. There is a relative depression in serum albumin and an apparent 'shift' in the positions normally occupied by the α- and β-globulins. The latter regularly appear to move as one band in electropherograms of sera from leukemic birds. It has been possible to induce this 'shift' in normal sera by storage or incubation.

Introduction

Little is known about the behavior of the serum proteins in the avian leukoses; because of the need of the proliferating leukemic cells to synthesize proteins, changes might be anticipated. In neoplasia in man, certain changes in the serum proteins, such as specific new proteins in the case of multiple myeloma, frequent elevation of the α_2-globulin in lymphocytoma and lymphoid leukemias, and increased α-globulin in myelogenous and monocytic leukemias have been reported (1).

Sanders *et al.* (2) studied serum and plasma from normal and leukosis-affected chickens using free electrophoresis. They found a new 'L' component closely associated with γ-globulin in samples from different forms of experimental and spontaneous leukosis. This, they believed, was of the nature of an antibody, although it did not appear as a separate component in electrophoresis of sodium-sulphate-precipitated γ-globulin obtained from leukotic birds. Krejci *et al.* (3), also using the Tiselius method, made similar studies on a transmissible fowl 'lymphoma' (RPL 16). They noted that rapid tumor growth was regularly accompanied by a reduction in albumin concentration, and that there was occasionally a reduction in globulin as well. The concentration of γ-globulin rose abruptly when tumor regression followed. Although they were aware of the work of Sanders *et al.*, they do not appear to have encountered a separable 'L' component.

Sharp *et al.* (4) have studied the electrophoretic properties of myeloblastosis virus. They used conditions almost identical with those of Sanders *et al.* The mobility of the virus was stated to be -4.5 ($\times 10^{-5}$ cm^2. sec^{-1} volt^{-1}), faster than Sanders' 'L' component, viz. -2.55.

The introduction of paper electrophoresis has made it much easier to obtain preliminary information on protein changes, and the technique has been applied in many instances to the study of neoplasia in man. To the writer's knowledge, the use of paper electrophoresis for the study of avian leukosis has been

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Contribution from the Animal Diseases Research Institute (Western), Health of Animals Division, Canada Department of Agriculture, Lethbridge, Alberta.

reported on only one occasion. Slizewicz and Atanasiu (5) noted no alteration of serum proteins in plasma from chickens with erythroblastosis; however, only small numbers of birds were employed. In contrast, we have made extensive studies on sera and plasma from birds with erythroblastosis, and have found definite and consistent differences.

Experimental

Reagents and Apparatus

Serum protein determinations were made in the first experiments using a modified (6) Weichselbaum reagent (7). A serum of known protein content (Kjeldahl) was used as a standard.

Two buffers were used for electrophoresis: barbiturate pH 8.6, ionic strength 0.075, and phosphate pH 6.4 (KH_2PO_4, $M = 0.055$; Na_2HPO_4, $M = 0.021$). A Durrum cell (Spinco) with S & S 2043A mgl. paper strips was used for paper electrophoresis and a photoelectric densitometer (Analytrol RB1, Spinco) for evaluating the stained strips.

Methods

East Lansing Line 15 White Leghorns were used at 5–8 weeks of age throughout these experiments. Preliminary observations showed that older birds were not very satisfactory, as poor separation of the serum fractions was obtained.

Blood was taken by heart puncture and sera were collected as soon as separation occurred, in all cases within 2–3 hours. Plasma was separated from citrated blood by centrifugation. To obtain leukemic sera and plasma, chicks were inoculated with an extract of erythroblastosis-infected liver known to produce 80% takes. The extract had been prepared by homogenization of the liver with 10 volumes of phosphate-buffered (pH 7.2) 6% dextrose and 5 volumes of Freon-heptane (3:1 w/w) (8). A Waring blendor run at half speed was used to homogenize the material; a 2-minute run was employed. The homogenized material was allowed to stand at 4° C for 3 hours and was then shaken and centrifuged in a Serval SS-1 angle centrifuge at 4200 r.p.m. for 15 minutes. The clear, red-colored supernatant constituted the extract used in these studies.

In most instances the course of the disease was followed by means of phase microscopy of unstained blood smears prepared daily after the 9th- to 10th-day postinoculation.

Sera were examined for their protein, glycoprotein, and lipoprotein content. The amount of sera applied to the paper strips varied with the technique, 0.02 ml, 0.05–0.10 ml, and 0.04 ml respectively, for the three procedures. Runs were for 16 hours at 2.5 ma current for the barbiturate and 5 ma current for the phosphate buffers.

For demonstration of the serum proteins with bromphenol blue, 'procedure B' (Spinco Manual RIM5) was used; for glycoproteins, a periodic acid – 'Schiff' technique modified from Grönwall and Köiw (9); and for lipoproteins, a saturated solution of Sudan black in 50% ethanol. In the latter procedure strips were stained for 10 minutes in the dye solution at 56° C, and then successively rinsed twice with cold 50% ethanol for 6- and 1-minute periods.

Results

Initial Experiments

Barbiturate and phosphate buffers were compared in studies on miscellaneous normal and leukemic sera which had been stored in bottles in a CO_2 ice chest. The relative amounts of the individual proteins present are given in Table I.

TABLE I

Percentages of serum protein in sera from normal and erythroblastosis-infected birds as indicated by paper electrophoresis

Buffer	Serum	Number examined	Serum protein, mg/ml	Albumin	Globulin	
					α and β	γ
Barbiturate	Normal	11	14.5±0.6	66.6±0.3	14.6±0.4	18.8±0.7
	Leukemic	12	13.8±1.2	39.2±1.7	27.7±0.9	33.1±1.4
Phosphate	Normal	10	14.9±0.6	48.5±0.7	24.5±0.9	27.0±0.5
	Leukemic	10	14.2±1.3	33.0±1.6	27.1±1.0	39.9±1.6

In leukemic sera, with both buffers, the level of albumin was lower and the globulin higher, as noted by Krejci *et al.* (3). A lower percentage of albumin was obtained with the phosphate buffer due to better separation of the α-globulin from albumin or resulting from some effect on the subsequent staining of the proteins. With phosphate buffer, the α- and β-globulins moved as a single component and did not move as far in leukemic sera. Normal sera examined with barbiturate buffer showed separation of the α- and β-globulins but these apparently merged in electropherograms of leukemic sera.

Determination of Time of Onset of the Serum Changes

Four 5- to 6-week-old birds were inoculated with erythroblastosis virus January 28, 1958, and four were used as controls. The eight birds were bled for sera from the wing vein January 27, 29, February 3, 5, 6, 7, and 8, 1958. Electrophoretic runs were again made with barbiturate and phosphate buffers. No important alterations occurred in the control sera (Table II). In the leukemic birds, the drop in albumin was not as marked as in the initial experiments, but the trend was still present. Similar changes to those observed in the initial experiments occurred (Fig. 1, A and B), but did not appear until 2 days before death. The α- and β-globulin 'shift' was again most pronounced with phosphate buffer (Fig. 1, C and D). Actual stained strips are shown in Fig. 2. In Table III the distance moved by the globulin peak is shown as a ratio of that moved by albumin. The figures indicate that the 'shift' only became significant on the last day of bleeding but a tendency for a 'shift' could be detected 2 days before.

Comparison of Plasma and Sera

Plasma was also investigated to determine whether the specific changes occurred during clotting. Two transmission experiments were made to provide plasma and serum for this purpose.

In the first experiment, 14 birds were inoculated with erythroblastosis virus; there were nine controls. Two of the inoculated birds died on the 10th day and

TABLE II

Serum protein changes with the development of erythroblastosis

Sera	Barbiturate buffer				Phosphate buffer		
		Globulins				Globulins	
	Albumin	α	β	γ	Albumin	α and β	γ
Control sera collected Jan. 27 to Feb. 8, 1958	60.4±1.0	6.6±0.2	13.7±0.4	19.2±0.8	46.2±0.8	25.5±0.6	28.3±0.2
Sera from infected birds collected Jan. 27 to Feb. 5, 1958	56.0±0.7	6.4±0.3	13.5±0.8	24.2±0.8	44.6±0.9	23.2±0.9	32.2±0.6
Sera from infected birds, Feb. 6, 1958	48.4±0.7	10.2±0.5	15.6±1.3	25.8±1.8	42.0±1.1	23.4±0.9	34.6±1.1
Feb. 7, 1958	50.3±0.8	25.4±1.3		24.2±1.4	38.4±1.6	29.0±0.6	32.7±1.5
Feb. 8, 1958	47.2±3.5	23.3±1.7		29.6±3.1	31.4±1.5	28.7±2.1	39.9±6.1

TABLE III

Distance moved by alpha–beta component as a ratio of that moved by albumin with progression of erythroblastosis

Date*	Distance moved	
	Controls	Inoculated
Jan. 27	0.52±0.02	0.49±0.03
Jan. 29	0.45±0.03	0.49±0.01
Feb. 3	0.55±0.02	0.50±0.03
Feb. 5	0.53±0.01	0.46±0.02
Feb. 6†	0.53±0.03	0.44±0.02
Feb. 7	0.54±0.01	0.44±0.03
Feb. 8	0.56±0.03	0.32±0.02

*The infected birds were inoculated January 28, 1958.
†Proerythroblasts were found in the circulating blood of one or more of the inoculated birds after this date.

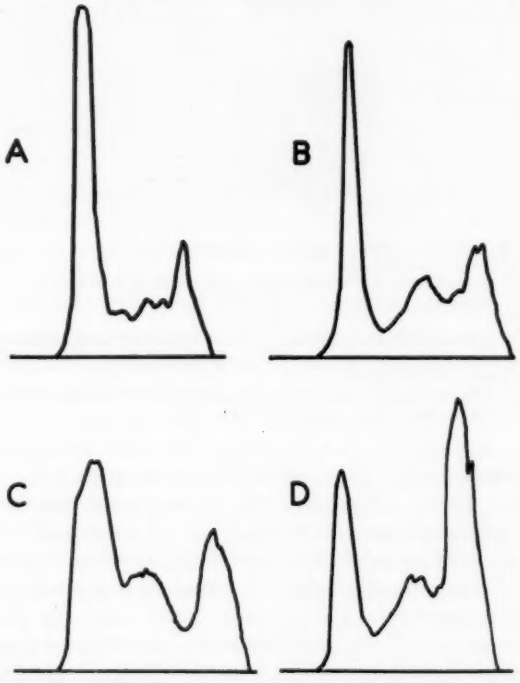

FIG. 1. Electrophoretic patterns of sera from control and erythroblastosis-infected chicks.

Barbiturate buffer: (A) normal, (B) infected.
Phosphate buffer: (C) normal, (D) infected.

plasma was collected from the survivors and the controls. Electrophoretic runs were made with the infected and normal plasma using phosphate buffer. The globulin 'shift' occurred only in those plasmas obtained from birds in which the disease was advanced as indicated by post-mortem appearance and the liver histology.

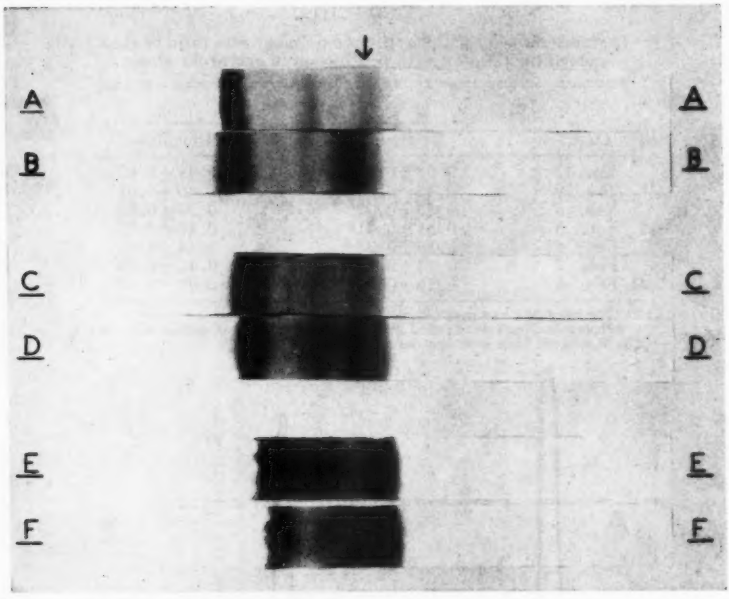

FIG. 2. Photograph of electropherograms; anode to the left.
Barbiturate pH 8.6 buffer
(A) Sheep serum for comparison with chick serum below.
(B) Note the similarity in general appearance with minor differences in banding.
(C) Second normal chick serum for comparison with leukemic serum below.
(D) Note 'merging' of α- and β-globulin bands.
Phosphate pH 6.4 buffer
(E) Normal chick serum for comparison with leukemic serum below.
(F) Note shift in $\alpha\beta$-globulin zone.

In the second experiment 27 birds were inoculated with the virus. There were 16 controls. When a leukemic picture developed, the birds were bled from the wing vein for plasma, from the heart for sera, and were then killed and autopsied. Four of the birds survived 14 days without becoming leukemic when plasma and sera were also collected. There was again a good agreement between the development of the globulin 'shift' and the development of leukemia. However, one of the 15 control sera also showed a suggestion of a 'shift'. There was a good agreement between patterns obtained with both plasma and sera.

Mucoproteins

No consistent differences were detected in studies on 15 normal and 21 leukemic sera and plasma using the Schiff reagent. In view of the largely glycoprotein nature of the globulins involved in the 'shift', marked changes with the Schiff reagent were expected. We prefer to regard our failure to demonstrate these as due to limitations of the technique, but it is possible that a non-glycoprotein is involved.

Lipoproteins

The position of Sudan IV staining bands in relation to bromphenol blue staining bands was determined for 11 leukemic and 5 normal sera following electrophoresis with the phosphate buffer. Most of the lipoprotein was retained as a wide band at the site of application. One sudanophilic band was also found in the albumin position and others occurred between the albumin and point of application. Since the latter did not necessarily correspond with the α- and β-globulin bands demonstrated with bromphenol blue, it would seem unlikely that lipoproteins are directly involved in the 'shift'.

The Effect of Dialysis

Since the plasma or sera used in the above experiments had not been subjected to dialysis, it was possible that the changes we had observed were not due to differences in the proteins, but to non-protein dialyzable fractions. Two normal and two leukemic sera in which the 'shift' occurred were used. They were each divided in half and one portion was dialyzed in viscose tubing for 2 hours at 4° C against a large excess of pH 6.4 phosphate buffer (two changes) before application to the strips. Dialysis for this time had no effect on the electropherograms obtained.

Denaturation

Early in these experiments the effect of the addition of hemoglobin to normal sera was investigated. It was found that the 'shift' could be produced in normal serum by incubation with hemoglobin. Later studies showed this effect to be variable and that it merely accelerated a normal process, namely, that the 'shift' could be produced by incubation of normal serum. The time of incubation required varied from a few hours to 2 days at 37° C or 3 hours at 57° C. This stressed the need for the quick collection of serum after clotting. It was also found that sera kept for a few days at 4° C or for several weeks at -15 to -20° C would eventually develop the 'shift'.

Denaturing processes other than incubation or storage that we investigated did not produce the characteristic 'shift'. For instance, mild oxidation of plasma with hydrogen peroxide caused remarkable changes in the protein patterns. There was an increase in the area occupied by α- and β-globulins at the expense of albumin, until the latter disappeared completely; however, the α- and β-globulin 'shift' towards γ-globulin was not observed.

Discussion

The lowered serum albumin and raised serum globulin observed by us in erythroblastosis agrees with the findings of Krejci *et al.* (3). Involvement of the liver sinusoids with proerythroblasts eventually develops in erythroblastosis which might lead to liver dysfunction and resultant alterations in the serum proteins. Since lymphoid tumor viruses capable of inducing erythroblastosis may produce cytopathogenic changes in cultures of chick embryo liver cells (10), and although similar properties have not yet been recorded for laboratory strains of erythroblastosis, it is possible that the latter may be pathogenic to liver cells.

The globulin 'shift' has some bearing on the pathogenesis of the disease and it will be important to determine whether the 'shift' also occurs in other forms of chicken and animal leukemias and other diseases. The 'shift' does not occur in birds with the Rous sarcoma (author's observation). We also need to know whether the 'shift' precedes or follows the blood changes, although our present observations would suggest the latter.

There are many possible explanations for the 'shift'. It might follow an absence or lowered level of α-globulin (1), but there was little difference in the level of the α- and β-globulins in the sera from the inoculated and control groups. The 'shift' might also occur through the combination of α-globulin fractions with other proteins (i.e. new proteins produced by the tumor cells) in the same way that the haptoglobins of man combine with hemoglobin (11). This supposition led us to investigate the effect of the addition of hemoglobin to normal serum and its incubation. We found that the incubation of normal serum alone induced the 'shift' but that the addition of hemoglobin could accelerate this process. Thus circulating hemoglobin must be considered a possible factor in the 'shift' and further investigations in this direction are required. Although Bonar and associates (12) find more hemoglobin in the plasma of birds with erythroblastosis, we have not been able to confirm this.

The 'shift' would appear to us to be the result of a form of denaturation of the α- and β-globulins. That this denaturation occurred in the filter paper is possible, but runs at 4° C on four leukemic plasma still showed the 'shift'. If denaturation occurs in vivo, profound physiological disturbances might be expected, since the α- and β-globulins are involved in so many important biological systems. We have, for instance, often noted slow clotting and lengthened prothrombin times on the part of leukemic bloods. Alterations of proteins involved in blood clotting may thus be involved in the 'shift'.

Published studies on disease in chickens using paper electrophoresis are limited, but Common *et al.* (13, 14) have reported in detail on healthy birds. They used long runs (24–30 hours) and obtained very clear separations but the probability of 'equilibrium' conditions (15) with our apparatus decided us against runs of that length. Our estimates of total serum proteins are lower than those of Vanstone *et al.* (14), but serum protein levels were approximately equal in leukemic and normal birds. Some very low values were found in leukemic birds in which ascites developed.

In conclusion, in spite of an earlier negative report (5), paper electrophoresis does disclose certain changes in serum and plasma proteins in birds affected with erythroblastosis. These changes require an explanation. They may be of a nonspecific nature but they could also have an important bearing on the etiology of erythroblastosis.

Acknowledgments

I am very gratetul to Dr. L. Niilo of this Institute for assistance with the preparation of the diagrams in this paper. My thanks are also due to Dr. W. P. McKinley, Department of National Health and Welfare, Ottawa, and Dr. R. J. Avery, Officer-in-Charge of this Institute, for advice and suggestions.

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NOTES

METABOLISM OF PROGESTERONE AND TESTOSTERONE BY MAMMALIAN CELLS GROWING IN SUSPENSION CULTURE

D. PERLMAN, P. W. JACKSON, NANCY GIUFFRE, AND JOSEF FRIED

In a recent publication Sweat *et al.* (1) described experiments showing that when progesterone-4-C^{14} was added to uterine fibroblasts (strain U12-705) growing in a nutrient medium on glass surfaces, some of the added steroid was converted to Δ^4-pregnene-20α-ol-3-one, Δ^4-pregnene-20β-ol-3-one, and allopregnane-3,20-dione, as shown by mobility of the labeled steroids in chromatographic systems. During the past few years, methods for growing mammalian cells in suspension culture have been described by Owens *et al.* (2), Cherry and Hull (3), McLimans *et al.* (4), and others. Many cell lines have been grown in suspension culture in rotating test tubes, stirred bottles, and agitated stainless steel tanks. We have studied the metabolism of progesterone and testosterone by the L cell line of mouse fibroblasts (a cell line of non-glandular origin) growing in 1-liter stirred glass bottles, and have isolated and characterized by chemical degradation the products from progesterone as Δ^4-pregnene-20α-ol-3-one and Δ^4-pregnene-20β-ol-3-one, and from testosterone as androstene-3, 17-dione.

The L cell line was grown in suspension in a modification of Ziegler's medium (5). Preliminary experiments showed that inclusion in the medium of 25 p.p.m. of progesterone reduced the growth rate significantly, and the following procedure was developed to obtain cells capable of growing in the presence of the steroid: Approximately 350 ml of sterile medium was added to a 1-liter sterile centrifuge bottle containing 300 μg of progesterone and a teflon-covered magnetic stirring bar. Approximately 35 million L cells were added from a 4-day-old culture and the unit placed on an induction stirrer located in a 37° incubator. After 3 days' incubation when the cell count had reached 175 million, 350 ml of fresh medium was added and incubation was continued for another 4 days with daily adjustment of pH (with sterile NaOH) to pH 7.3. At this time, when the cell count was approximately 490 million, 15 mg of sterile progesterone was added (as a dry suspension) and the incubation continued for another 3 days. At the end of this interval the cells and liquid were extracted twice with ½ volumes of methyl-*iso*-butyl ketone. The extracts were pooled and concentrated to about 10 ml *in vacuo*. A small aliquot was examined by filter paper partition chromatography using Carbitol as the stationary phase and methylcyclohexane as the moving phase. The steroids present were detected by the Haines and Drake ultraviolet scanner (6). In addition to progesterone, two metabolites with mobilities of about 0.45 and 0.35 that of progesterone were detected in significant quantities. Similar samples taken from experiments where progesterone was mixed with the medium ingredients (but the L cells were not added) showed only progesterone. Approximately 80% of the progesterone added to the L cell suspension was not metabolized

by the cells. The rest of the extract was fractionated by large-scale paper chromatography using the above system and the two metabolites obtained in chromatographically pure form. The faster moving one had a mobility equivalent to that of Δ^4-pregnene-20α-ol-3-one. After oxidation with chromic acid it had a mobility about equal to that of progesterone, and its acetate had a mobility about 1.4 times that of progesterone. The slower moving fraction was tentatively identified as Δ^4-pregnene-20β-ol-3-one on the basis of its mobility in the paper chromatographic system with a standard sample of Δ^4-pregnene-20β-ol-3-one, the mobility of its acetate, and the identification of the chromic acid oxidation product with progesterone in two paper chromatographic systems. To confirm these findings the steroid present in approximately 12 liters of cell culture (representing about 200 mg of progesterone) was fractionated by the above procedure. The chromatographic fraction thought to contain Δ^4-pregnene-20β-ol-3-one (23.2 mg) when purified further by recrystallization from acetone–benzene had a m.p. of 171 to 173° (a standard sample of Δ^4-pregnene-20β-ol-3-one had a m.p. of 173°) and an infrared spectrum (KBr) identical with that of authentic samples of Δ^4-pregnene-20β-ol-3-one. Oxidation of the isolated crystalline product (7 mg) in acetone (2 ml) with chromic acid reagent (0.26 ml) (containing 20 mg CrO_3 and 32 mg H_2SO_4 in 1 ml of a mixture of 1 volume of water and 9 volumes of acetone) furnished progesterone (5 mg) possessing a melting point of 126° and an infrared spectrum identical with that of an authentic sample.

Similar experiments were carried out with testosterone. The preliminary study showed that only metabolite with an $R_{\text{testosterone}}$ equal to that of Δ^4-androstene-3,17-dione was formed. The steroidal metabolites present in 4 liters of fermented medium (representing about 80 mg of testosterone) were extracted with methyl-iso-butyl-ketone as described above, purified by paper chromatography, and the extracts evaporated to dryness *in vacuo*. The residual crystalline material (113 mg) had a melting point of 156 to 158°. Chromatography on acid-washed alumina afforded 56 mg of essentially pure Δ^4-androstene-3,17-dione, m.p. 172 to 174°; $(\alpha_D)^{23}$ +185° (*c*, 0.95 in EtOH). Its infrared spectrum (Nujol) was identical with that of an authentic sample. Control experiments where testosterone was added to the uninoculated medium showed that no transformations took place in the absence of the L cells.

These experiments show that this cell line of non-glandular origin carries out the same types of transformations previously found in the uterine fibroblasts, liver, and other tissues. They show that the enzyme systems found in non-glandular cells are capable of transforming steroids.

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A SIMPLE AUTOMATIC STIMULATION TIMER

G. CONSTANTOPOULOS

We have recently developed an automatic timing device which can be used in experiments where repeated electrical stimulation is required. By virtue of its simplicity it poses no servicing problem and it is inexpensive compared to the automatic timers available commercially. Furthermore it may be constructed from the equipment ordinarily available in the laboratory.

Description

The circuit between the stimulator and the electrodes is interrupted by two parallel triangular insulated copper plates held on a laboratory stand. The shaft

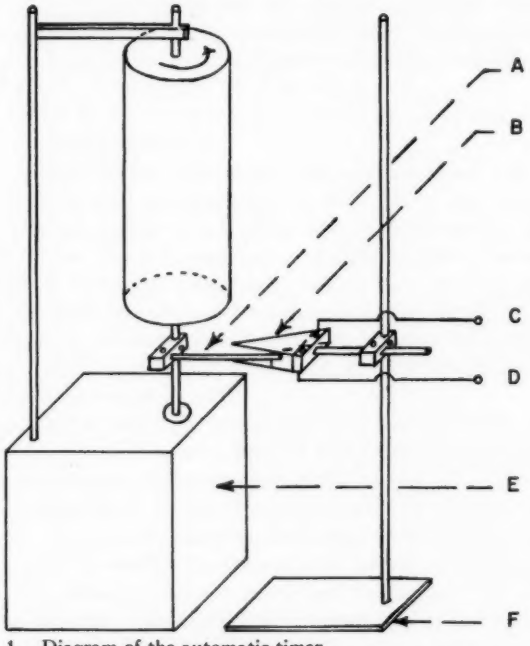

FIG. 1. Diagram of the automatic timer.
A. Plastic rod, $\frac{1}{4}$ in. diameter, with conducting strip.
B. Parallel copper plates attached to insulating block.
C. To stimulator.
D. To electrode.
E. Kymograph with variable speed gearbox.
F. Laboratory stand.

of a kymograph is used as a driving device for a plastic stem carrying a flexible phosphobronze strip. When the kymograph rotates, the strip comes into contact and brushes the copper plates through part of the circle it describes. With the stimulator switched on, current will pass and electrical stimulation will be delivered only while the strip is in contact with both plates. The frequency of the periods of stimulation can be adjusted by changing the speed of the kymograph and the duration of these periods can be controlled simply by moving the triangular metallic plates closer or further away from the revolving plastic stem (see Fig. 1).

Since no permanent alteration of the kymograph is necessary it can easily be used again for its original purpose when the timer is no longer needed.

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Symposium on Some Biochemical Aspects of the Cancer Cell

This symposium was held on June 10, 1959, at the Second Annual Meeting of the Canadian Federation of Biological Societies at the University of Toronto, Toronto, Ontario. Dr. J. H. Quastel, of the McGill–General Hospital Research Institute, acted as Chairman and Editor of the symposium, which was organized and sponsored by the Canadian Biochemical Society.

Introductory remarks of the Chairman, as well as the first and third papers, are published as delivered at the symposium. The second paper has been expanded into a review article under the same subject heading.

INTRODUCTORY REMARKS

J. H. QUASTEL[1]

The Symposium, this afternoon, is to be devoted to some biochemical aspects of the cancer cell.

Our speakers are Dr. Morgan, who will deal with the subject of amino acid metabolism in normal and malignant cell cultures and Dr. Nichol, who will deal with the topic of metabolism of neoplastic cells in relation to drug response. Finally, there is a joint paper by Drs. Siminovitch and Axelrad, who will cope with biological problems in cancer biochemistry.

I have been asked to say a few words by way of an introduction to the subjects of this afternoon's symposium.

Biochemical aspects of the cancer cell are now widening and commanding more attention as it is becoming evident that there are other interesting features to be studied besides the implications of high aerobic glycolysis and the peculiarities of the tumor's respiratory processes. Our interest in the many biochemical aspects of tumors arises not only because we wish to know more of the manner in which the chemical machinery of the cancer cell differs from that of normal adult, or embryonic, cells but because we realize that these differences may yield a rational approach to the all-important practical problem of control of cancer growth.

Recent experimental work (1) has shown that tumors share with embryos the property of being much more effective than most normal adult tissues in synthesizing their proteins at the expense of the energy made available to them by respiration or glycolysis. In fact under anaerobic conditions, in the presence of glucose, when tumors and embryos synthesize their proteins approximately as well as under aerobic conditions, the majority of normal adult cells, including regenerating liver cells, are almost powerless to do so. But this similarity between the biochemical characteristics of tumor and embryo does not extend very far. A significant difference between these two tissues is the fact that tumors can bring about protein synthesis in the presence of potent respiratory inhibitors so long as glucose is present, a property not displayed by the embryos so far investigated. Presumably a drug that can abolish this activity in the

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presence of glucose has potentialities for cancer control, if its toxicity to normal tissues is not too high.

The evidence obtained so far provides good support for the conclusion that adenosine triphosphate is the major energy factor in the process of amino acid incorporation into the proteins of cells. Apart from the supply of energy, made available either by respiration or by aerobic or anaerobic glucose breakdown, a specific chemical factor is the supply of glutamine, the high glutaminase activity of tumors making the availability of exogenous glutamine an important factor in its metabolism and growth. Probably the presence of other amino acids is equally important. Aside from these, we have to consider the fundamental role of the nucleic acids in the establishment of protein synthesis and specific effects of substances such as guanosine triphosphate, which is held to play a catalytic role in transferring an activated amino acid to the nucleoprotein.

Another phenomenon which is receiving more consideration in cancer biochemistry is the progressive increase of high anaerobic and aerobic glycolytic rates of mammalian cells with time of *culture in vitro*, as, for example, rabbit kidney cells in the experiments of Warburg *et al.* Experiments carried out by Dr. R. Johnstone in our Institute show that the "altered monkey heart" cells of Salk, kindly made available to us by the Connaught Medical Research Laboratories, have biochemical characteristics in vitro, similar to, if not identical with, those of the tumor cells we have examined. But what are these cells? Are they truly heart tissue cells that have become changed, or dedifferentiated, by continued subculture, or are they contaminating tumor cells that have developed rapidly, to the exclusion of the heart cells, under favorable nutritional and environmental conditions? Must normal mammalian cells invariably need the stimulus of an invading (virus) nucleic acid to show eventually, after continued subculture, the biochemical characters of tumor cells or may the stimulus arise in some other way?

I have not had the advantage of seeing in advance this afternoon's communications, so I do not know if my introduction is relevant to the topics to be discussed or not. I know, in any event, that the subjects of our speakers are of great importance to all of us and we look forward to them with much interest and pleasure.

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AMINO ACID METABOLISM OF NORMAL AND MALIGNANT CELL CULTURES[1]

JOSEPH F. MORGAN AND ARTHUR E. PASIEKA

A major objective in studies on the nutrition and metabolism of tissue cultures has been to discover differences between normal and malignant cells, with the hope that such differences might make it possible to inhibit the growth of malignant cells through the use of specific analogues. While this objective has always been implicit in tissue culture studies, it has received increasing emphasis during the past few years with the development of new and more precise methods of cell cultivation. In this presentation, I would like to discuss our own studies on the amino acid metabolism of normal and malignant cell cultures and to describe one apparently consistent difference in regard to the metabolism of glutamine.

In undertaking comparative studies on normal and malignant cells, the question immediately arises "How does one define a normal cell" and "what conditions are necessary to maintain tissue cultures in a 'normal' state". Before undertaking comparative studies, it is also necessary to have a background of information on normal cells so that a baseline for comparison is available.

It has been recognized recently that nearly, if not all, established cell strains in tissue culture exhibit malignant characteristics, particularly in regard to number of chromosomes and ability to form tumors in cortisone-treated animals. This generality appears to hold true whether the cell lines are derived from apparently normal tissue or from malignant tissue. After a number of serial subcultivations, the cell lines appear remarkably similar and *all* appear to have some malignant characteristics. The one common feature so far apparent is that development of these cell lines has involved continuous and rapid propagation.

In establishing our criteria for "normal" cultures, accordingly, we have selected fresh tissues as our starting point, and have *avoided* both subcultivation and rapid propagation. Our nutritional experiments are based on length of survival of our cultures, under conditions which maintain active metabolism. While this method imposes certain limitations, we believe that it has allowed us to accumulate valid information on the nutrition and metabolism of "normal" cultures, and to establish a reference baseline for comparative studies with malignant cultures.

For such comparative studies, it is, of course, essential that approximately similar conditions be employed with the malignant cells. For this reason, our investigations with the established cell strains have been carried out with

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moderately heavy cell populations which are increasing only slowly, but which are actively metabolizing.

Our experimental program has been built upon the use of completely synthetic media, since we believe that precise information on cell cultures requires precise control of the culture environment. We prepare variations in synthetic media, more than 2500 to date, and study the effect of these media on the survival of our cultures. At the same time, we remove the used culture media and examine them for changes in amino acid content. In this way, we obtain the effect of the medium upon the cells and also the effect of the cells upon the medium. We consider that this combined approach allows us to evaluate the over-all relationship of the cell to its environment.

Methods

Tissue culture is primarily a method, and there are innumerable variations that have been developed for specific purposes. In this presentation, I do not propose to discuss methods in detail but should mention briefly the general techniques we have used.

Our nutrition experiments have been carried out almost entirely with freshly explanted chick embryonic heart tissues cultivated in test tubes by the roller-tube method (1, 2). In these studies, we employ a nutritional depletion technique (3) which increases the response of our cultures to essential factors. Our

TABLE I

Composition of synthetic medium 199*

	mg per liter		mg per liter
L-Arginine	70.0	Thiamin	0.010
L-Histidine	20.0	Riboflavin	0.010
L-Lysine	70.0	Pyridoxine	0.025
L-Tyrosine	40.0	Pyridoxal	0.025
DL-Tryptophan	20.0	Niacin	0.025
DL-Phenylalanine	50.0	Niacinamide	0.025
L-Cystine	20.0	Pantothenate	0.010
DL-Methionine	30.0	Biotin	0.010
DL-Serine	50.0	Folic acid	0.010
DL-Threonine	60.0	Choline	0.500
DL-Leucine	120.0	Inositol	0.050
DL-Isoleucine	40.0	*p*-Aminobenzoic acid	0.050
DL-Valine	50.0	Vitamin A	0.10
DL-Glutamic acid	150.0	Calciferol	0.10
DL-Aspartic acid	60.0	Menadione (K)	0.01
DL-Alanine	50.0	α-Tocopheryl PO_4	0.01
L-Proline	40.0	Ascorbic acid	0.05
L-Hydroxyproline	10.0	Glutathione	0.05
Glycine	50.0	Tween 80 (oleic)	20.0
Cysteine	0.1	Cholesterol	0.20
Adenine	10.0	Sodium acetate	50.0
Guanine	0.3	L-Glutamine	100.0
Xanthine	0.3	ATP	1.0
Hypoxanthine	0.3	Adenylic acid	0.2
Thymine	0.3	Ferric nitrate	0.1
Uracil	0.3		
Ribose	0.5		
Desoxyribose	0.5		

*This medium is made up in a modified Tyrode's solution, containing glucose, with a final concentration of 1.5 mg % phenol red indicator.

metabolism studies (4–6) have been carried out with a variety of freshly explanted tissues and established cell strains cultivated in T-flasks. These cell strains include the L strain, HeLa cell, human amnion, and three lines of human leukemic cells.

In our metabolism experiments (4–6), we have relied most heavily upon the techniques of paper chromatography. Our methods have been developed around two solvent systems, and both general and specific amino acid reagents. In all, we apply about seventeen different color tests to our chromatograms. We employ both one-dimensional and two-dimensional techniques.

All our nutrition and metabolism experiments are based on the use of our synthetic medium, which has the formula listed in Table I. This medium contains both the essential and the non-essential amino acids, vitamins, purines, pyrimidines, and certain accessory growth factors, including glutamine. It is prepared in a modified Tyrode's solution, containing glucose (1, 7).

Results

A typical nutrition experiment, using chick embryonic heart cultures, is illustrated in Figs. 1–6, and shows representative areas from six equivalent cultures. In the complete medium (culture 1) extensive sheets of large, spindle-shaped cells can be seen; in the absence of tyrosine (culture 2) only a few small degenerating cells are visible; in the absence of arginine (culture 3) only small granular cells are apparent; in the absence of glutamic acid (culture 4) extensive sheets of large, healthy cells can be seen which compare favorably with those in the complete medium; in the absence of cystine (culture 5) the cells are granular and degenerating; in the absence of valine (culture 6) only rounded granular cells can be seen.

From this type of experiment, it has been possible to determine the effect of omitting each of the individual amino acids from the synthetic medium, as summarized in Fig. 7 (8). The data in this graph are calculated as percentage differences from the control cultures. Consequently, bars extending to the left indicate amino acids required by the cultures, while bars to the right indicate amino acids that are not required. The graph, as a whole, shows the essential and non-essential amino acids for chick embryonic heart cultures.

Experiments in our own and many other laboratories have established the amino acid requirements of a number of cell types (9). These are summarized and compared in Table II. It is evident that the amino acid requirements of most cell types, of both normal and malignant origin, are essentially similar. This picture may change as more detailed information is accumulated but, at the present time, nutritional studies alone have not uncovered major differences between normal and malignant cells in tissue culture. For that reason, we considered it necessary to switch our investigations to a detailed study of the amino acid *metabolism* of normal and malignant cells.

Our first efforts were devoted to determining metabolic patterns in a variety of freshly explanted normal tissues cultivated in our completely synthetic medium. In Fig. 8 is shown the pattern of changes in the amino acid content of

TABLE II

Comparative amino acid requirements of various types of tissue cultures and of man

Amino acid	Chick fibroblasts	L strain	HeLa strain	Carcinoma Walker 256	Rabbit fibroblasts	Man
Arginine	+	+	+	+	+	−S
Histidine	+	+	+	+	+	−
Lysine	+	+	+	+	+	+
Tryptophan	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+
Tyrosine	+	+	+	+	+	−S
Cyst(e)ine	+	+	+	+	+	−S
Methionine	+	+	+	+	+	+
Serine	−	−	−	−	+	−
Threonine	+	+	+	+	+	+
Leucine	+	+	+	+	+	+
Isoleucine	−	+	+	+	+	+
Valine	+	+	+	+	+	+
Glutamic acid	X	−	−	−	−	−
Aspartic acid	X	−	−	−	−	−
Alanine	X	−	−	−	−	−
Proline	X	−	−	−	−	−
Hydroxyproline	X	−	−	−	−	−
Glycine	−	−	−	−	−	−
Glutamine	−	+	+	+	+	
Asparagine				+		

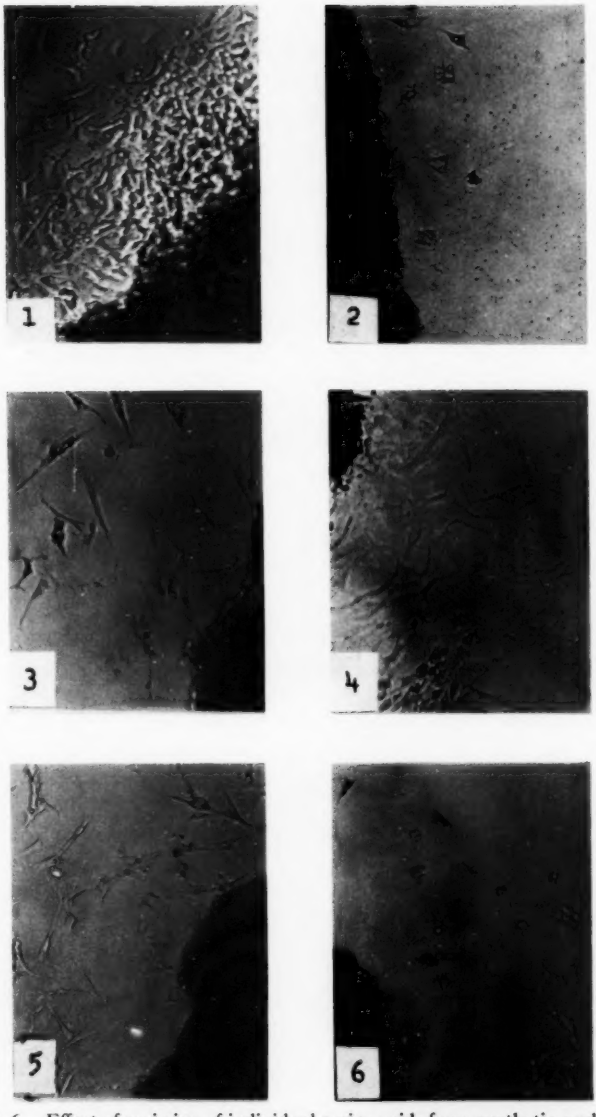
\+ Indicates essential.
− Indicates non-essential.
S Indicates stimulating.
X Indicates inhibitory.

TABLE III

Comparative pattern of changes in amino acid composition of synthetic medium M 150 during cultivation of various tissues in vitro[a]

Amino acid	Chick heart	Chick liver	Chick kidney	Monkey kidney
Cystine	***	**	**	**
Histidine	**	*	*	**
Arginine	***	*	*	**
Tyrosine	*	*	*	*
Tryptophan	**	*	**	*
Valine	**	*	*	*
Isoleucine	**	**	**	*
Leucine	**	**	**	*
Serine	+	+	+	+
Glycine	+	+	+	+
Threonine	++	++	++	++
Alanine	+++	+	++	++
Methionine	+++	+	+	++
Glutamine	+++	++	++	+
Lysine	**	+	+	+
Aspartic acid	*	+	0	0
Hydroxyproline	*	0	0	0
Glutamic acid	*	+	0	++
Proline	+	+	**	*
Phenylalanine	**	**	**	0

[a] Changes based on the most active period of metabolism of each culture type.
\+ = Slight increase, ++ = moderate increase, +++ = marked increase.
* = Slight decrease, ** = moderate decrease, *** = marked decrease.
0 = No detectable change.



FIGS. 1–6. Effect of omission of individual amino acids from synthetic medium 199 on morphology of chick embryonic heart cultures. All cultures photographed at 5 days. Culture 1, complete synthetic medium; 2, tyrosine omitted; 3, arginine omitted; 4, glutamic acid omitted; 5, cystine omitted; 6, valine omitted. Magnification, 105×.

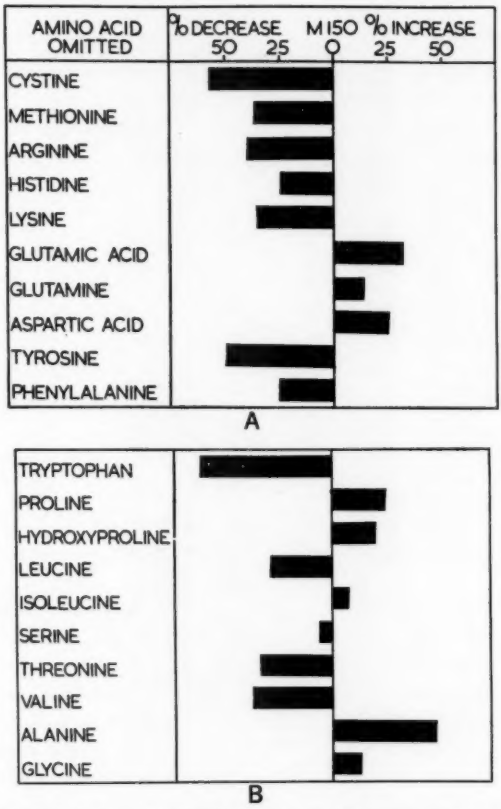


FIG. 7. Effect of omission of individual amino acids from synthetic medium 199 on survival of chick embryonic heart cultures. All values expressed as percentage differences from the survival time of control cultures in complete medium.

the culture medium during cultivation of chick embryonic heart tissues. In this chromatogram, which illustrates the degree of separation and resolution we normally obtain, the even-numbered spots represent used culture media while the odd-numbered spots represent unused control media. It is apparent that extensive changes have occurred in the medium during tissue cultivation.

Extension of these studies to a variety of freshly explanted tissues established that each tissue investigated brought about a slightly different pattern of amino acid decrease and accumulation in the culture fluids. This is summarized in Table III. It should be noted that all these cultures were consistent in increasing the glutamine content of the culture medium.

Comparative studies were next undertaken with a series of cell lines of malignant origin. The results obtained with strain L mouse cells are shown in Table IV. The first column illustrates the pattern of changes in the complete synthetic medium, which contains both glutamic acid and glutamine. The

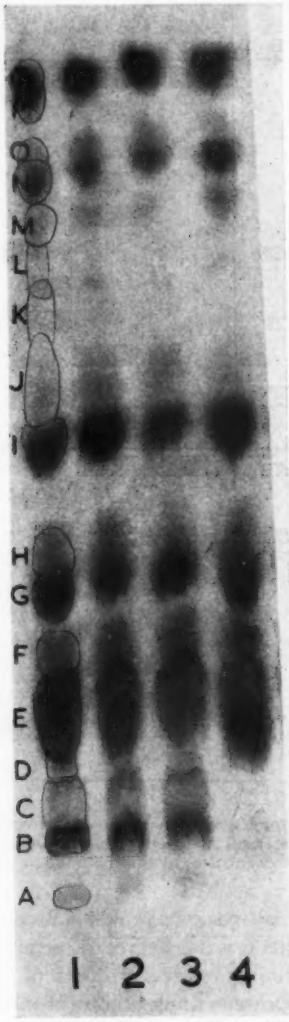


FIG. 8. Amino acid changes in synthetic medium 199 during cultivation of chick embryonic heart tissues. Chromatogram developed with butanol – acetic acid – water mixture and sprayed with ninhydrin. Nos. 1 and 3 = unused medium controls. Nos. 2 and 4 = medium removed from cultures 1 week after explanation, and on 19th day of cultivation, respectively.

Q Isoleucine
P Leucine
O Phenylalanine
N Valine
M Methionine
L Tryptophan
K Tyrosine
J Proline
I Alanine
H Threonine
G Glutamic acid
F Glycine
E Aspartic acid
Serine
Hydroxyproline
D Glutamine
C Arginine
B Lysine
Histidine
A Cystine

TABLE IV

Summary of changes in amino acid composition of various synthetic media during cultivation of L strain cells[a]

Amino acid	Cultivation medium			
	Complete medium	Glutamic omitted	Glutamine omitted	Glutamic, glutamine omitted
Cystine	**	**	**	*
Lysine	*	*	**	0
Histidine	**	**	**	0
Arginine	*	*	**	0
Glutamine	**	**		
Aspartic acid	0	0	0	0
Serine	+	+	0	0
Hydroxyproline	0	0	0	0
Glycine	+	+	0	0
Glutamic acid	++	++	**	++
Threonine	+	+	*	0
Alanine	0	0	**	0
Proline	+	+	**	0
Tyrosine	+	+	*	+
Tryptophan	*	*	0	0
Methionine	++	++	*	0
Valine	*	*	**	0
Phenylalanine	**	**	**	0
Isoleucine	*	*	**	*
Leucine	**	**	**	*

[a] * = Uptake. 0 = No change. + = Accumulation.

second column shows that omission of glutamic acid does not change this pattern appreciably. Omission of glutamine (column 3) greatly increases amino acid uptake, expecially that of the non-essential amino acids. Omission of both glutamic acid and glutamine (column 4) causes a virtual cessation of all amino acid changes in the culture medium. From Table IV it is apparent that strain L cells *remove* glutamine from the culture fluids and that this amide plays an important role in amino acid metabolism. It was of particular interest to note that the cultures did not die even though amino acid changes ceased (6). Glucose was still metabolized and uptake of adenine proceeded at an apparently normal rate.

In Fig. 9 are shown changes in the glutamine area of the synthetic medium during cultivation of the HeLa strain of human malignant epithelial cells. Two-dimensional chromatographic techniques were employed in these experiments. It is evident that glutamine (region 5) has been almost completely removed from the medium, and that the content of glutamic acid (region 10) has been considerably reduced.

Since the HeLa cell, and many other malignant cell lines, do not propagate extensively or even survive for long periods of time in the completely synthetic medium, extensive studies were made with and without a supplement of human serum. With all cell lines tested, the pattern of amino acid changes was found to be identical whether or not serum was present. In the presence of serum, however, the pattern of changes persisted for a much longer period than in its absence. From these experiments, it was concluded that short-term studies in completely synthetic media provided valid data on the amino acid metabolism of malignant cell strains.

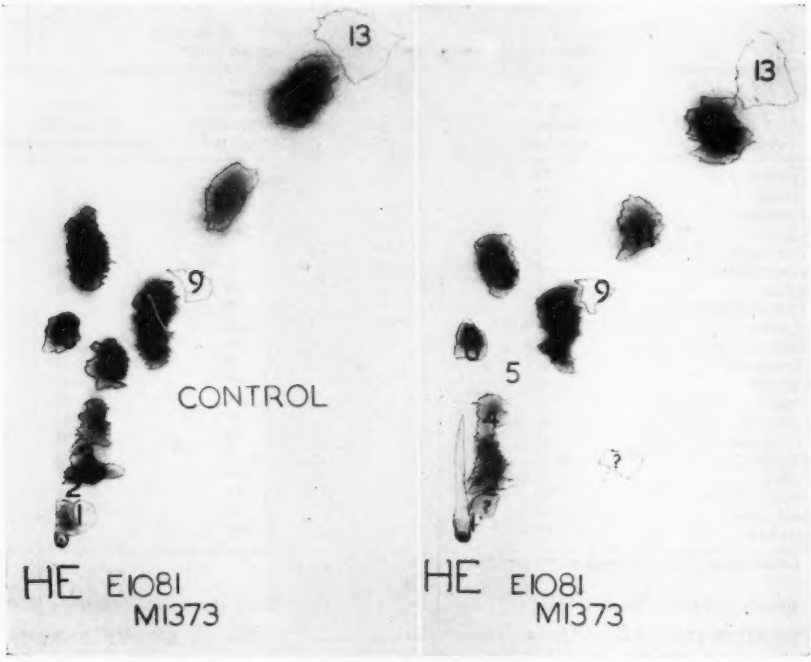


FIG. 9. Amino acid changes in completely synthetic medium during cultivation of strain HeLa cells of human malignant epithelium. Chromatogram developed with butanol – acetic acid – water mixture and sprayed with ninhydrin.

1. Cystine
2. Lysine
3. Histidine
4. Arginine
5. Glutamine
6. Aspartic acid
7. Serine
8. Glycine
9. Hydroxyproline
10. Glutamic acid
11. Threonine
12. Alanine
13. Proline

TABLE V

Changes in glutamine concentration of synthetic medium during cultivation of various cells and tissues

Tissue cultures	Change in glutamine content of medium[a]
Chick embryonic heart	+++
Chick embryonic kidney	++
Chick embryonic liver	++
Monkey kidney explants	+
Monkey kidney trypsinized	*
Strain L (mouse fibroblasts)	**
HK 55 (human kidney)	**
FL strain (human amnion)	**
Strain HeLa (human malignant epithelium)	***
J-96 (human leukemic)	***
J-111 (human leukemic)	***
J-128 (human leukemic)	***

[a] Changes are based on most active period of metabolism of each culture type.
+ Indicates increase.
* Indicates decrease.

Our studies to date on glutamine are summarized in Table V. It is evident that the freshly explanted tissues cause a marked increase in the glutamine content of the medium. On the other hand, the established cell strains, which are largely of human malignant origin, bring about a marked decrease in the glutamine concentration. This decrease is most marked with the HeLa strain and the three lines of human leukemic cells. The behavior of monkey kidney cells appears to depend on the method of preparation of the cultures. Primary cultures of freshly explanted fragments cause a slight increase in glutamine content of the medium, while primary cultures prepared from a trypsinized cell suspension cause a slight decrease in glutamine concentration.

Discussion

Our combined nutrition and metabolism studies on normal and malignant cells over the past several years appear to have established several points, which I would now like to summarize briefly:

(1) Studies in our own and other laboratories have not shown major differences in amino acid requirements between normal and malignant cell cultures. Recent studies on carbohydrates have indicated that different cell types may vary in their ability to utilize certain sugars but it is too early to determine whether these differences are characteristic of the malignant and non-malignant cell.

(2) The pattern of amino acid changes in the culture medium appeared to be qualitatively similar whether or not the synthetic medium was supplemented with serum. This similarity was found to exist with all the cell lines so far tested, including the HeLa strain, three human leukemic strains, a human amnion, and a human kidney strain. The effect is demonstrated to a very marked degree with the human leukemic cells, which degenerate and die within 3 weeks when the cultures are maintained in the synthetic medium but survive and propagate when a human serum supplement is provided. Even under such rigorous conditions, the pattern of amino acid change in the culture medium appears qualitatively identical. Our results to date suggest that this similarity of pattern is a general phenomenon and is not restricted to any one cell type.

(3) The major finding of our investigations has been the observation that cultures of freshly explanted tissues accumulate glutamine in the surrounding medium while all established cell strains tested remove this amide from the culture fluids. This appears to establish a consistent metabolic difference between freshly explanted tissues and established cell strains, which were predominantly of human malignant origin. The present observation may be interpreted, accordingly, either as a possible metabolic difference between normal and malignant cells, or as a possible alteration in cell metabolism associated with the establishment of permanent cell lines in tissue culture.

The observation that established cell strains develop malignant characteristics makes it appear hopeful that tissue culture techniques may help to solve this basic problem of malignancy and we are now studying cultures in the

intermediate period between the explantation of the original tissue and the emergence of established cell strains.

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METABOLISM OF NEOPLASTIC CELLS AND THEIR RESPONSE TO CERTAIN DRUGS[1]

CHARLES A. NICHOL

The effectiveness of chemotherapy depends on the selective action of a drug such that target cells are affected without adverse effects on normal tissues of the body. The basis for the selective effects of chemotherapeutic agents is understood in relatively few cases and the elucidation of the mode of action of drugs on specific metabolic pathways or individual enzymes presents a continuing challenge to biochemists and pharmacologists. The action of certain agents which are curative in some infectious diseases can be attributed to interference with metabolic processes peculiar to the susceptible organism. For example, sulphonamides impair the utilization of a compound (*p*-aminobenzoic acid) which is a vitamin for certain bacteria but not for mammalian cells (47, 97, 98). Penicillin interferes with the synthesis of constituents of bacterial cell walls (uridine pyrophosphate N-acetyl-muramic acid peptides) which are not present in mammalian cells (66, 67). Although limitations in treatment may be imposed by the insolubility of some sulphonamides which can result in deposition of crystals in the renal tubules or by a hazard associated with an acquired sensitivity to penicillin, the mode of action of these agents on infectious organisms is not related to their toxic effects in man. The common use of antibiotics in media for the culture of mammalian cells emphasizes this distinction. Since these drugs act on metabolic targets in the parasite which are not present in cells of the host, the selective action is sufficient to achieve a curative effect.

Unfortunately, the action of drugs which have shown moderate effectiveness in the treatment of certain types of cancer is not limited to neoplastic cells. This is indicated in many cases by damaging effects on cells in the bone marrow and intestinal epithelium. Limitations in dosage imposed by the susceptibility of normal cells and the consistent development of drug resistance in malignant cells following successful remissions require classification of present anticancer agents as palliative rather than curative.

The extension of therapy by chemicals beyond Ehrlich's original concept of killing infectious organisms invading the body (19, 55) to treatment of neoplastic cells produced by the body requires recognition of certain limitations. The basis for any selective action in the latter instance is more restricted since neoplastic cells have much the same composition and complement of enzymes to be found in other tissues of the body. The defense mechanisms which can cope with large numbers of invading organisms are of limited effectiveness against cells produced by the body. Thus, a curative anticancer agent must

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eliminate most, perhaps all, neoplastic cells. Failure to do so may be an important factor in permitting the selection and outgrowth of cells refractory to treatment. Although no agent can yet be described as curative of cancer in man, instances of remission of leukemias and certain solid tumors induced by antimetabolites and alkylating agents have been associated with complete disappearance of all symptoms of the disease (22). Unfortunately, relapse has occurred after varying periods. These observations, however, demonstrate the potentialities of such chemotherapy. The incidence of acute and chronic leukemias and of the metastatic forms of solid tumors which cannot be treated by surgery or radiation adds urgency to the search for new drugs and the finding of means to increase the effectiveness of presently useful agents.

Three different approaches to a common objective characterize current work in the field of cancer chemotherapy. (i) Random screening of chemicals and biological materials against arbitrarily selected transplantable tumors in rodents applies the concept that no stone should be left unturned in this search for new drugs. A major program of this type sponsored by the United States Public Health Service now has the capacity to test some 25,000 materials each year (14). Most of the active compounds found to date fall within classes of presently known effective agents. A relatively high proportion of fermentation broths (approximately 2%) inhibits the experimental tumors. After careful screening, such new antibiotics will receive increasing attention. A number of new compounds from different sources are now undergoing clinical trials. (ii) A different approach of most interest to pharmacologists concerns the description of the sites of action of known anticancer agents, the nature of resistance to these drugs, and means of modifying their physiological disposition or achieving increased effectiveness by combining different treatments. Can the selective action of these agents, demonstrated so strikingly by instances of complete remissions, be duplicated or extended to drug-refractory neoplasms? (iii) The work most challenging to biochemists concerns the elucidation of the differences between neoplastic and normal cells. These cells are different in many respects. Kit and Griffin (45) have emphasized in a recent review that populations of neoplastic cells are characterized by profound aberrations with respect to the function and morphology of the cell nucleus. Studies by Hauschka (38) on the genetics of neoplasia have directed attention to many individual characteristics of tumor cells. Can detailed understanding of differences in metabolism make possible the chemical poisoning of neoplastic cells without harming normal tissues or can metabolism be altered in a manner converting a malignant to a benign tumor? The establishment of a relationship between viruses and tumors in animals and in man will undoubtedly influence each of these three approaches to cancer chemotherapy (57).

It is appropriate to emphasize the relationship of biochemical characteristics of neoplastic cells to potential chemotherapy. Many investigations of biochemical differences between normal and malignant cells have disclosed quantitative rather than qualitative enzymatic features (29, 85). The discovery by Warburg (89) of the high rate of anaerobic glycolysis of tumor cells has remained for many years as an outstanding biochemical characteristic of neoplastic cells

(90, 94). Weinhouse has presented evidence that such cells have no defect in oxidative metabolism although a quantitative difference in glycolysis is a consistent characteristic (93, 94). Control of this altered metabolism has been attempted through the use of compounds capable of inhibiting hexokinase (12). Another characteristic of tumor metabolism concerns a shift in the balance of anabolic and catabolic pathways which may be related to the capacity of neoplastic cells for growth. In a discussion of the enzyme-deletion hypothesis of the origin of cancer, Potter (69) cited 14 catabolic enzymes which are low or missing in the Novikoff hepatoma. The detailed studies of DeLamirande, Allard, and Cantero (17) and of Weber and Cantero (92) on the distribution and level of enzymes in normal and tumor tissues have contributed notably to such comparisons. The observation by Boxer and Shonk (10) that α-glycerophosphate dehydrogenase in an induced tumor as well as in a large series of transplantable tumors is either absent or low as compared with a variety of normal tissues may be of fundamental significance in characterizing the metabolism of neoplastic cells. The reverse situation which might be more amenable to direct chemotherapeutic attack, namely, the occurrence of an enzyme in neoplastic cells that is absent from normal tissues, has not been reported. This is the type of observation, however, that may form a basis for more effective chemotherapy. Such investigations should be interpreted where possible in the light of our rapidly increasing knowledge about the fine structure of cells. Recent electron microscopy disclosing the endoplasmic reticulum and various cell compartments indicate the complex relationships between structure and function of cells (82). The study of factors which limit the growth of organs may lead to the identification of growth regulatory substances. If one kidney is removed, the other increases in size and functional competence. Upon partial hepatectomy, the remaining tissue proliferates only until hepatic capacity is restored. During regeneration of liver in rats, the mitotic rate of other tissues is increased and implanted tumors grow more rapidly (64, 65) suggesting the presence of a humoral agent. Biochemical studies of such experimental material may yield information applicable to the control of neoplasia.

The diverse chemical agents which can inhibit the growth of tumors can be grouped, with minor exceptions, into three main classes of compounds, namely, alkylating agents, steroids, and antimetabolites. A number of new potent compounds have been added to each of these groups recently. The literature describing the diverse effects of these drugs is now voluminous, if not overwhelming (13). A recent publication listed more than 400 *review* articles on cancer research within a 5-year period (40). Several recent articles have reviewed special aspects of the metabolic effects of these drugs (26, 35, 54, 56, 58, 83). For the purpose of this presentation, I propose to select two drugs, an antimetabolite and a steroid, which are being studied in our laboratories at the Roswell Park Memorial Institute to illustrate the manner in which drugs can alter the metabolism of neoplastic cells. Before proceeding to a discussion of these two examples, several details of current interest concerning the alkylating agents can be mentioned briefly.

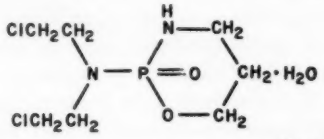
Alkylating Agents

The application of these chemicals to the treatment of cancer grew out of studies on the toxic effects of mustard war gases. These compounds, now a heterogeneous group, are highly reactive and combine with so many cellular substances that the detection of the locus of their action is difficult (2, 74, 79). These drugs are sometimes referred to as radiomimetic agents because of the similarity in their effect on cells to the damaging result of ionizing radiation (3, 70, 84). Considerable evidence indicates that DNA is a target of the alkylating agents, and two hypotheses suggest that "crosslinking" of DNA (3, 4, 84) or the reactivity of ring-N of the constituent bases (50, 73) is responsible for the action of these compounds. Biologically active alkylating agents of the N-mustard or ethyleneimine type react with the deoxyribonucleic acid in the cell nucleus to form crosslinked structures which swell, but no longer dissolve in concentrated solutions of sodium chloride. Alkylating agents which are not cytotoxic do not produce this effect. Correlation of crosslinking in vitro with activity in vivo is more satisfactory in the case of N-mustard compounds than with bifunctional epoxides and esters of methane sulphonic acid, such as Myleran. Crosslinking between adjoining structures could involve reaction with the phosphodiester groups of DNA or alkylation of the bases of DNA. Under physiological conditions, the most reactive groups of DNA appear to be the ring-nitrogen atoms of guanine, adenine, and cytosine. This reaction is followed by cleavage of the bases from the deoxyribose phosphate linked chain. The loss of guanine which occurs to the greatest extent may be attributed to the higher reactivity of the 7-position (50, 73). Such cleavage of the bases would be expected to impair the functional integrity of the DNA molecule with effects which could be independent of the occurrence of crosslinking. In studies which can provide one measure of the functional capacity of DNA, it was found that alkylation of relatively few of the bases in transforming principle interfered with the transmission of characteristics (73). Reactivity with a small number of bases or linking of relatively few twin strands of DNA by an alkylating agent might profoundly alter capacity for cell division. It is likely that the reactivity of these compounds with DNA is also related to their carcinogenic (88) and mutagenic effects (6).

These alkylating agents can be considered to be composed of two functional structures, a reactive portion which combines with tissue substances and a carrier portion of the molecule which can influence the physiological disposition of the compound. Bergel (8) has discussed the design of alkylating agents with respect to selectivity of action. Investigations now in progress in several laboratories will provide data making possible a better comparison of different compounds based on the ratio of toxic to effective doses in animals bearing experimental tumors. Preliminary results indicate highly significant differences in the relative effectiveness of a variety of alkylating agents (80).

An increase in the selective action of alkylating agents might be achieved if the neoplastic cells have greater enzymatic capacity to convert an inactive to an active compound. Acylation decreased the toxicity of some aromatic N-mustards for rats; correlation was noted between the presence in a trans-

plantable tumor of an enzyme capable of splitting off the acyl group and the inhibitory action of the drugs (39). A potent new compound (Endoxan, Cytoxan) of considerable interest in present clinical studies is a diphosphoramide internal phosphoric acid ester of nitrogen-mustard (5).



Cyclophosphamide
(Endoxan, Cytoxan)

This compound is relatively inert in vitro. Phosphamidases, which have been reported to occur in malignant tissue in increased amounts (27), presumably hydrolyze the cyclic group at the phosphorus–nitrogen bond thereby allowing ionization of the chlorines in the chloroethyl groups. In rats treated with Endoxan the diphosphopyridine nucleotide level of tumor tissue was lowered; the administration of nicotinamide prevented both the lowering of the DPN level and the inhibition of tumor growth (41). Such observations may reveal the metabolic sites most sensitive to the effects of alkylating agents and may lead to the availability of more effective compounds.

Antimetabolites

Two drugs of this class which are used in the treatment of leukemia (22) are amethopterin (Methotrexate, 4-amino-10-methyl pteroylglutamic acid) and 6-mercaptopurine (Purinthol); the former is closely related to the structure of the vitamin, folic acid (pteroylglutamic acid), and the latter can be considered to be an analogue of hypoxanthine. It seems to be characteristic of antimetabolites that relatively small structural differences from the corresponding metabolites can confer remarkable changes in biological potency. In addition to the above examples, it may be noted in this respect that 5-bromouracil deoxyribonucleoside acts as an analogue of thymidine (20, 32, 44), whereas the very similar compound 5-fluorouracil deoxyribonucleoside interferes with the utilization of uracil deoxyribonucleoside (9, 16). Among the various analogues of naturally occurring compounds that have been tested as inhibitors of growth in different systems, a very high proportion are analogues of components of nucleic acids or can be shown to interfere with the biosynthesis of nucleic acids (35). It is more than mere coincidence that potent inhibitors, as different in structure as 5-fluorouracil deoxyribonucleoside and amethopterin both interfere with the biosynthesis of thymidine, a unique component of DNA. Such drugs are useful tools in the study of biosynthetic pathways and investigation of their action discloses metabolic sites where interference by drugs can be most critical to cell survival.

Earlier discussion emphasized the desirability of finding qualitative differences in metabolism with respect to potential cancer chemotherapy; it should be made clear, however, that the activity of the above antimetabolites as

anticancer agents demonstrates that quantitative differences in metabolism *can* serve as a basis for the selective inhibition of growth. For example, folic acid cofactors are essential for the biosynthesis of thymidine, purines, and a number of amino acids. This vitamin is a useful drug for the treatment of certain megaloblastic anemias; its function is also vital to the growth of neoplastic cells, and folic acid antagonists, such as amethopterin, can achieve partial control of certain types of tumors and leukemias. In addition to the remarkable remissions in some cases of acute leukemia in children (22), effective treatment with amethopterin of choriocarcinoma in women has been reported more recently (51, 52). In other types of neoplasms in man, however, the effectiveness of this agent has been quite limited. Detailed studies of effects of antimetabolites on metabolic processes are cited in recent review articles (35, 54, 56, 83). Current studies on the site of action of amethopterin and means of circumventing the inhibitory effect of the drug on the growth of cells in culture will be discussed as an example of experiments attempting to understand more completely the action of one antimetabolite. Most of the experiments which I will now discuss were carried out by my colleagues, Dr. Maire Hakala, Dr. Fred Rosen, and Dr. William Werkheiser, and I am indebted both to their willingness to allow me to present some unpublished observations and to their stimulating association in the progress of this work.

Inhibition of Folic Acid Reductase by Folic Acid Antagonists

An indication of the enzymes inhibited by these antagonists was given by the finding that the conversion of folic acid by rat liver slices to tetrahydro derivatives measurable as folinic acid was inhibited by aminopterin (62). Subsequent investigations have shown that the specific reactions blocked by antagonists of this type are the formation by folic acid reductases of the dihydro and tetrahydro forms of pteroylglutamic acid (25, 63, 68, 99). N^{10}-Formyl, N^{10}-hydroxymethyl, N^5–N^{10}-methylene derivatives of the tetrahydro form appear to be the cofactors catalyzing the transfer of C-1 units in different biosynthetic reactions (37, 42). The toxic effects of these antagonists on bone marrow and intestinal epithelium, as well as on cancer cells, can be attributed to a specific interference with the formation of the functional forms of folic acid.

Firm binding of amethopterin in tissues was indicated by the inability of folic acid or its derivatives to prevent the toxicity of this drug for mice when administered following treatment (28) and by its prolonged retention in tissues of the mouse (24). The nature of the binding of amethopterin by folic acid reductase has been shown by recent studies by Werkheiser (96). The amount of amethopterin required to completely inhibit this enzyme was 0.56 μg/g of rat liver; supernatant fractions from livers of rats treated with supralethal doses of amethopterin contained (16 hours after injection) 0.52 μg of amethopterin per g of tissue. Extensive dialysis (three 48-hour dialyses against 1000 volumes) with buffered sucrose solutions failed to remove more than a small fraction of the drug from the supernatant preparation; dialysis against relatively high concentrations of pteroylglutamic acid (1 mg/ml) in sucrose completely

removed amethopterin. In similar experiments using aminopterin-2-C^{14}, 85% of the radioactivity was dialyzed out in the presence of pteroylglutamic acid and 85% of the folic acid reductase activity was restored. The "irreversible" nature of the binding of the drug to the enzyme is shown by the data in Fig. 1;

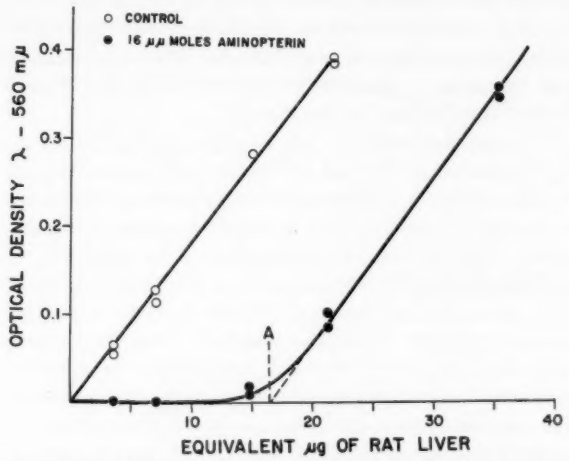


FIG. 1. Irreversible inhibition by aminopterin of folic acid reductase in rat liver supernatant. The reaction mixture, which was incubated at 37° for 20 minutes, contained 40 mμmoles of pteroylglutamic acid, 10 mμmoles of TPNH, 5 μmoles of $MgCl_2$, 5 μmoles of citrate, 50 μmoles of dimethylglutarate buffer of pH 6.1, and rat liver supernatant fraction in a total volume of 0.5 ml. The diazotizable amine derived from tetrahydropteroylglutamic acid was determined colorimetrically. From "A" it is calculated that the supernatant from 1 g of liver binds 440 mμg of aminopterin. (W. C. Werkheiser, unpublished data.)

in the presence of a small amount of the drug, no activity is apparent when increasing amounts of the enzyme are used until the amount of enzyme is sufficient to bind all of the drug present. Thereafter, increasing amounts of enzyme increase activity in a manner which parallels the control preparations. Thus, this irreversible inhibitor can be titrated with the enzyme, applying a technique proposed by Ackermann and Potter (1), and this type of inhibition can be clearly distinguished from reversible inhibition (in the presence of a constant amount of a reversible inhibitor, the activity is proportional to the amount of enzyme). The binding of amethopterin by folic acid reductase, which cannot be reversed by physiologically attainable concentrations of folic acid, appears to account for the amount of the drug retained in tissues. There are few examples of such correlation between the binding of a drug and its mode of action.

New Nutritional Requirements for the Growth of Neoplastic Cells in the Presence of Amethopterin

On a theoretical basis, if a growth inhibitor acts by preventing the synthesis of a metabolite needed at some step in a sequence of biosynthetic reactions, then the effect of such a drug should be circumvented by making available that

compound which is a product of the drug-inhibited reaction. This has been shown to be the case in a number of studies using microbial techniques and the "inhibition analysis" proposed by Shive (81) was based on such interference with inhibition of growth. On a practical basis, the identification of metabolites which can prevent the inhibitory action of a drug can indicate not only its site of action but also compounds whose availability can be critical to growth. In other words, the presence of such compounds could limit the potential effectiveness of the agent. The following observations by Hakala and Taylor (34) are of special significance in this regard.

Cultures of mammalian cells do not require any purine or pyrimidine for growth in Eagle's medium (18) but the presence of some form of folic acid is essential. If this medium is supplemented with glycine, thymidine, and one of the naturally occurring purines, the growth of mouse Sarcoma-180 cells or human carcinoma (HeLa) cells is independent of the presence of this vitamin (34). Under these conditions, amethopterin is ineffective as an inhibitor, even at 10,000 times the concentration that inhibits growth of the cells in the unsupplemented medium (30). Thus, the addition of amethopterin to the culture medium creates new requirements for hypoxanthine or adenine, thymidine, and glycine, metabolites which are products of the drug-inhibited metabolic pathways. This demonstration that changes in the composition of the medium can condition the response of these cells to amethopterin directs attention to the availability of purines or thymidine to neoplastic cells in vivo as factors which could limit the initial effectiveness of amethopterin or influence the development of drug resistance.

Remissions are induced by amethopterin in only about one-half of the cases classified as acute leukemia of childhood. Even in instances of prolonged remissions induced by amethopterin, a drug-refractory condition eventually occurs. Although wide ranges in concentration of the drug may be studied in vitro (23, 30) it should be emphasized that toxicity limits dosage in vivo in a manner such that relatively small differences in dosage (four- or five-fold) may separate sensitive from drug-resistant neoplasms (60). Current investigations of drug resistance reveal changes in metabolism associated with the altered response. Since detailed discussion of this pertinent subject cannot be presented here, attention is directed to several publications (11, 49, 61, 72, 95).

The new requirements for the growth of mammalian cells in vitro in the presence of amethopterin make possible an extension of these experiments to studies on the mode of action of other antimetabolites. The complete dependence of growth on a source of purines and thymidine in this amethopterin medium make it suitable for the study of antagonists related to these metabolites (32, 33).

Cell Culture Studies on the Mode of Action of 6-Mercaptopurine

The effect of 6-mercaptopurine on the growth of Sarcoma-180 and HeLa cells was studied (i) in the folic acid medium in which all purine containing compounds in the cells are synthesized *de novo* and (ii) in the supplemented amethopterin medium in which the growth of the cells is dependent on the

presence of some preformed purine. The growth of S-180 cells was hardly affected even by high concentrations of 6-mercaptopurine when the amethopterin medium contained the minimum concentration of adenine which could support optimum growth; in the presence of hypoxanthine about 100 times more 6-mercaptopurine was required for inhibition of the growth of HeLa cells than in the folic acid medium (Fig. 2).

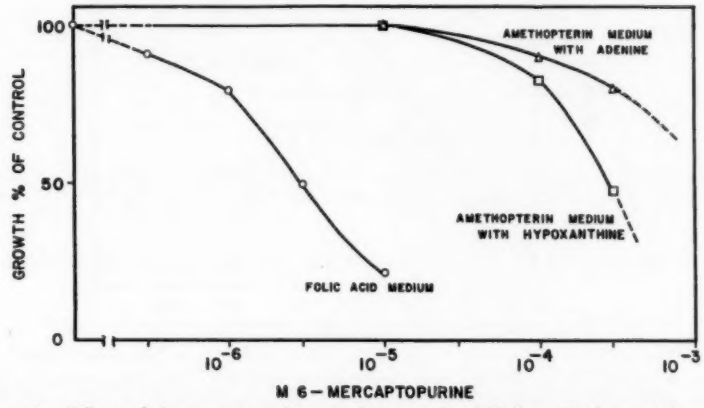


FIG. 2. Effect of 6-mercaptopurine on the growth of HeLa cells in varied media. ○——○ Eagle's medium with 10% horse serum; □——□ and △——△, Eagle's medium supplemented with 10^{-6} *M* amethopterin, 3×10^{-5} *M* thymidine, 3×10^{-4} *M* glycine, and 3×10^{-5} *M* hypoxanthine or adenine, respectively.

In the folic acid medium, constant concentrations of adenine, adenosine, or adenylic acid prevented the inhibitory effect of 6-mercaptopurine in a manner that was independent of the concentration of the drug. Hypoxanthine, inosine, and inosinic acid prevented the effect of 6-mercaptopurine in a manner that depended entirely upon the relative concentrations of drug and metabolites. Since synthesis of purines *de novo* occurs to an unknown extent in cultures growing in the folic acid medium supplemented with varied amounts of hypoxanthine, the competitive relationship between 6-mercaptopurine and hypoxanthine was analyzed in the amethopterin medium. An application of the inhibition analysis of Lineweaver and Burk (53) to this relationship is presented in Fig. 3. In this case the value for the initial reaction velocity (v) has been replaced by the ratio of the total protein after 1 week's incubation to that of the inoculum. On the basis of the analysis some apparent constants, corresponding to Michaelis constants, can be calculated (1.7×10^{-5} *M* for hypoxanthine and $9.3\times10^{-5}M$ for 6-mercaptopurine). The individual values of these constants must be accepted with reservations applying to the conditions used. The ratio of these constants, however, is significant in demonstrating that with HeLa cells the concentration of hypoxanthine required to nullify an effect of 6-mercaptopurine is only one-fifth that of this drug. The affinity of hypoxanthine derivatives for the site concerned appears to be about five times as great as that of 6-mercaptopurine. In the folic acid medium, the concen-

tration of newly formed inosinic acid is apparently quite low and thus, only low concentrations of 6-mercaptopurine are necessary for inhibition of growth.

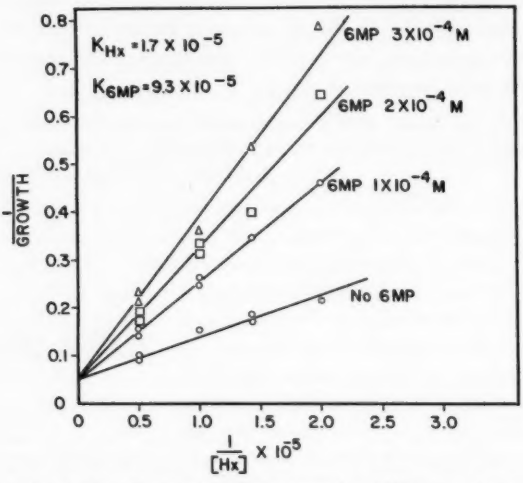


FIG. 3. Analysis of the effects of 6-mercaptopurine (6MP) on the growth of HeLa cells in amethopterin medium (Lineweaver and Burk). In the graph, growth is defined as total protein relative to inoculum after 1 week's incubation and [Hx] as molar concentration of hypoxanthine in the medium.

The inhibition of S-180 and HeLa cells by 6-mercaptopurine and also by its ribonucleoside was prevented by hypoxanthine, inosine, and inosinic acid in a competitive manner. Since inosinic acid but not hypoxanthine or inosine is on the pathway of purine synthesis *de novo*, the results of these experiments imply that 6-mercaptopurine and its ribonucleoside act in the form of the ribonucleotide by competing with inosinic acid on the pathway to adenylic acid (33). Further studies are needed to relate inhibition of growth to interference by the drug in this manner with the formation of nucleic acids or some essential cofactor containing adenylic acid.

Combinations of 6-Mercaptopurine with Amethopterin

The possibility that combination of a folic acid antagonist, such as amethopterin, with 6-mercaptopurine would result in a synergistic inhibition of the growth of mammalian cells in culture was investigated in the following experiment. The effects of both drugs on S-180 cells were studied alone and in combinations over concentrations ranging from 10^{-9} to 10^{-7} M for amethopterin and from 10^{-7} to 10^{-4} M for 6-mercaptopurine. The successive concentrations of each compound differed from each other by a factor of 3. Each of these concentrations was tried with all of the concentrations of the other compound. Figure 4 presents only the combinations that caused 50% inhibition of growth and each point was determined from a separate growth curve based on triplicate determinations. This type of analysis has been used in microbial studies to distinguish synergistic inhibition from a simple additive effect (21). No

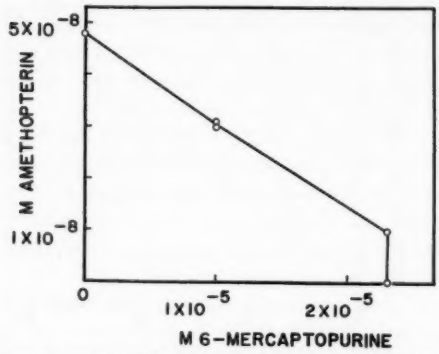


FIG. 4. The effect of combinations of 6-mercaptopurine and amethopterin on the growth of S-180 cells in folic acid medium. The points denote the concentrations of inhibitors required for 50% inhibition.

synergism could be detected; the effect of the combinations was only additive (33).

Selective Effect of a Thymidine Analogue on Deoxyribonucleic Acid

In the modified culture medium containing amethopterin, the normal growth of mammalian cells is dependent upon a source of thymidine. If this compound is omitted from the medium, the cells do not grow and soon disintegrate (34). In this respect, the result of thymidine depletion in the case of these mammalian cells differs from that observed in bacterial studies of "thymineless death" (15). Hakala (31, 32) observed that replacement of thymidine by 5-bromodeoxyuridine in the amethopterin medium permitted limited *growth* of several mouse and human cell strains; cultures of HeLa cells were able to double their content of DNA and complete one cell division. Thereafter, the synthesis of protein continued for several weeks (Fig. 5) even though the total DNA as well as the content of DNA per cell remained constant. The cells became greatly enlarged and eventually disintegrated. Isolation and analysis of DNA from these cells showed that DNA-thymine was replaced by 5-bromouracil to the extent of 50%, indicating that 5-bromodeoxyuridine was used for the synthesis of new DNA (32). Thus, the phenomenon of "unbalanced growth", i.e., an abnormal increase in the ratio of protein to DNA, occurs also in mammalian cells in a manner that parallels closely this phenomenon in bacterial cells (15). This unbalanced growth can be attributed to incorporation of the analogue into DNA in a manner that prevents further formation of DNA but does not prevent continuing synthesis of cellular protein. The interference with DNA metabolism by compounds which simulate thymidine or interfere with its biosynthesis is a subject under active investigation in several other laboratories (15, 16, 20, 44, 71).

Influence of Corticosteroids on Transaminase Activity

Hormones, like other drugs, exert their effects by modifying existing biochemical or physiological mechanisms rather than through the creation of any new function. Relatively small changes in the distribution of substrates within

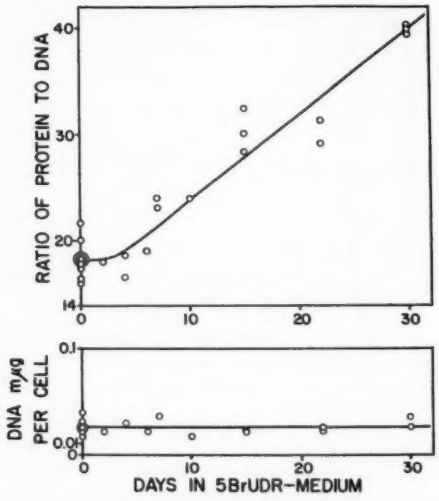


FIG. 5. The effect of 5-bromodeoxyuridine on the DNA content per cell and on the ratio of protein to DNA of HeLa cells growing in Eagle's medium supplemented with amethopterin (10^{-6} *M*), hypoxanthine (3×10^{-5} *M*), glycine (3×10^{-4} *M*), and 5-bromodeoxyuridine (3×10^{-5} *M*).

cellular compartments or in the rate of their utilization may have profound effects on the function or growth of tissues (43). Few enzymes are influenced directly in vitro by the presence of a hormone; the action of estradiol on the transfer of hydrogen between two pyridine nucleotide coenzymes (86) and on a DPN-specific isocitric dehydrogenase (87) in preparations from human placenta is of current interest with respect to possible correlations with the biological effects of estrogens. The extensive analysis by Mueller and his associates (59) of metabolic changes in estrogen stimulated uterine tissue may be cited as a logical biochemical approach attempting to decipher the mode of action of estrogens.

Many enzymes have altered levels of activity in tissues following varying periods of treatment with corticosteroids (46); with the exception of glucose-6-phosphatase (91) and glutamic pyruvic transaminase (78), however, it is difficult to relate the function of such enzymes to the biological role of these steroids. A more comprehensive discussion of the metabolic effects of these drugs would be appropriate to our topic. I can present here only a few observations from an investigation with Dr. Rosen of an effect of corticosteroids on the activity of two transaminases. Several features of this relationship are noteworthy. (i) The activity of glutamic pyruvic transaminase is increased 5- to 12-fold in liver of rats treated with cortisol without significant effect on the closely related glutamic oxaloacetic transaminase (76, 78); similar findings have been reported by Beaton and his associates (7). (ii) All of the compounds tested to date which have corticosteroid activity induce this response; other steroids devoid of gluconeogenic activity are inactive in this respect (77). (iii) The

activity of glutamic pyruvic transaminase is increased by treatment with cortisol in the thymus gland, and in experimental tumors which respond to this hormone (77). (iv) The activity of this enzyme in liver is decreased following adrenalectomy (36). (v) Other conditions associated with gluconeogenesis, namely, starvation, alloxan-diabetes, or feeding high protein diets, also increase the activity of glutamic pyruvic transaminase in rat liver without a corresponding change in the activity of glutamic oxaloacetic transaminase (78). Changes in the activity of glutamic pyruvic transaminase are closely associated with the response to corticosteroids and may condition an increased rate of gluconeogenesis.

In young rats implanted with Walker carcinoma 256, large doses of cortisol markedly impaired the growth of this tumor and increased the activity of glutamic pyruvic transaminase (Table I). Treatment with deoxycorticosterone

TABLE I

The effect of cortisol and deoxycorticosterone acetate (DOCA) on the glutamic pyruvic transaminase activity and growth of Walker carcinoma 256

Treatment,* mg/kg/day		Tumor growth†		Glutamic pyruvic transaminase activity (mmoles substrate utilized/g protein/hr at 38°)	
		g	% control		Treated/Control
None	—	4.2 ±1.3†	—	0.32± .06‡	—
Cortisol	10	1.5 ±0.45	36	1.4 ±1.0	4.4
Cortisol	30	0.35±0.07	8	4.7 ±1.6	14.8
Cortisol	50	0.41±0.07	10	4.6 ±1.2	14.4
DOCA	30	6.2 ±1.7	148	0.15± .06	0.47

*Compounds were injected subcutaneously for 14 days; treatment began 24 hours after the tumor was transplanted.
†The animals were sacrificed and the tumors weighed 15 days after transplantation.
‡Average values ± standard deviation. There were five rats in each group.

acetate (DOCA) appeared to have the opposite effect; in this case the activity of this enzyme was lowered and the rate of growth of the tumor was increased (Table I). This observation is of particular interest in view of the reported stimulation of the growth of a cortisone-sensitive mouse lymphosarcoma by treatment with DOCA (48).

If changes in the activity of this transaminase correlate with changes in rate of growth of the tumor, we can now ask, "Will conditions which induce gluconeogenesis and influence the activity of this transaminase in the same manner as treatment with cortisol also impair the growth of a cortisone-sensitive tumor?" This was found to be the case. In rats which were made diabetic by treatment with alloxan or in rats which were fed diets containing high levels of protein, the rate of growth of Walker carcinoma 256 was greatly reduced (75). The relation of glutamic pyruvic transaminase activity to increasing levels of dietary protein in adrenalectomized rats (78) indicates that more than an adrenal-mediated response can be involved. When the level of protein was increased to 50% of the diet, marked reduction in tumor size occurred at the same time that a normal rate of growth was maintained. The change in the activity of glutamic pyruvic transaminase in this experiment was considerably

less than that resulting from treatment with cortisol (75). Similar inhibition of tumor growth was observed in rats which were fed high levels of protein, made diabetic, or treated with cortisol. The stimulation of tumor growth by DOCA which was accompanied by a decrease in the activity of the pyruvic transaminase adds significance to this correlation. These preliminary experiments outline an approach toward an understanding of the relationship between alterations in transaminase activity and the growth of a cortisone-sensitive tumor.

These studies on the relationship between corticosteroid treatment and transaminase activity or on factors modifying the effects of amethopterin illustrate that metabolism can be altered in a manner permitting correlation of biochemical changes and growth. There is a basis for optimism that progress toward the control of neoplastic growth can be accomplished by the identification and exploitation of those aspects of metabolism most essential for cell growth.

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SOME BIOLOGICAL PROBLEMS IN CANCER BIOCHEMISTRY[1]

LOUIS SIMINOVITCH AND ARTHUR AXELRAD

Abstract

Understanding of the cancer process in chemical terms has been seriously hampered by the difficulty of interpreting results of biochemical comparisons between masses of tumor and of normal tissue. Normal tissue consists of a variety of cell types and tumors may originate from one or more of these. As whole masses, therefore, normal tissues cannot serve as adequate controls for experiments on any single tumor. Tumor cell populations, even those arising from a single cell type, are themselves cytogenetically and continually undergoing changes during growth (progression). It is thus difficult, if not impossible, to separate the relevant from the irrelevant biochemical features of malignancy.

Progress in this field requires means of dealing with the problem of biological heterogeneity. Several biochemical approaches that are free from the hazards of heterogeneity and which have already yielded valuable results, or appear promising, are indicated. These include: (1) The use of ascites tumors for studying the biochemical machinery of cells. No normal tissue exists, however, that could serve as satisfactory control. (2) Biochemical comparisons between pairs of tumor lines which differ by only one inherited characteristic of malignancy. These might reveal a biochemical basis for the biological properties of tumor cells with different degrees of malignancy. (3) Elucidation of normal growth-controlling mechanisms between cells, e.g. action of hormones at the cellular level, and within cells, e.g. mechanism of feed-back control of enzymes and metabolic pathways. (4) Further research into the biochemistry of plant tumor induction in vitro. Here biochemical changes associated with inherited changes leading to nutritional autonomy and uncontrolled growth have already been demonstrated. (5) Studies on the biochemical events during induction of malignancy by viruses in clonal cultures of animal cells in vitro. These could serve as useful models of the whole process of carcinogenesis.

If a single cell from an ascites tumor is injected into the peritoneal cavity of a young mouse, about once in every seven times on the average the cell will grow progressively, invade the adjacent organs, spread to and colonize other parts of the body, and cause the death of the mouse within 3 weeks. Clearly the whole of the capacity for malignancy can reside in a single cell and each time that cell divides, it can pass on this capacity to its daughter cells.

This inherited derangement in the ability to respond to the normal growth-controlling influences of the body must have its basis in profound alterations in the complex organization of enzymatic reactions of the cell. It follows that in order to understand what is happening in the cancer cell we have to know much about its basic biochemistry. Our knowledge of this is still fragmentary and we obviously need many more facts. However, it may be that even if we accumulated many more facts of the kind that we have now, it might still not be possible to work out a rational explanation of the cancer process in biochemical terms.

This is perhaps an unnecessarily pessimistic view. Even if it is, it might help to take stock of some of the present directions in cancer biochemistry in the light of the more recent advances in knowledge of the biology of tumors and see whether any useful applications can be made. A number of biologists

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and biochemists including Furth (1), Klein (2), and Potter (3) have been concerned with some aspects of these problems in recent reviews.

The modern era of cancer biochemistry began more than 30 years ago when Warburg (4) demonstrated that the malignant tumors he examined were all similar to each other in having relatively high rates of aerobic glycolysis and relatively low respiratory rates. From these facts Warburg developed the broad concept that an alteration in oxidative metabolism is the essential feature of all cancer cells and that a defect in respiratory metabolism is the basic cause of malignancy (4, 5). Although many biochemists did not accept this hypothesis, they did continue to use his technique of comparing a large variety of tumors with normal tissues in the hope of finding one or more common and unique biochemical defects responsible for malignancy.

As difficulties in interpretation of the results became apparent, various refinements in the experimental design were introduced such as the use of regenerating and embryonic tissue as controls along with normal adult tissue. And changes were made in the method of expressing results; for example, the "per cell" basis began to be used instead of "per mg wet or dry weight of tissue". Still no causal connection could be established between the metabolic derangements observed and the ability of the cell to grow progressively, to spread in and to kill its host.

Most biochemical studies, for technical reasons, have required the use of fairly large masses of tumor and normal tissue. One of the major limitations of these materials is their inherent heterogeneity. If the basic change in neoplasia were an absolute loss or gain of a single biochemical component, then this heterogeneity of normal and tumor tissues would not interfere with the search for the key alteration. But if, as seems likely from studies to date, the changes in the important components are multiple and relative, then heterogeneity would introduce an unknown variable into the results which could make them uninterpretable.

We shall be concerned here with biological heterogeneity, how it can interfere with the interpretation of biochemical results and how attempts have been made to solve this problem by careful selection and control of the biological material. We shall then indicate what in our minds appear to be areas of research where co-operation between the biologists who prepare and control the material and the biochemists who analyze it may further our understanding of the cancer cell.

The heterogeneity of any normal organ is evident histologically. Daoust (6) finds that the normal adult rat liver is composed of about two-thirds hepatic cells and one-third littoral cells. Bile duct cells comprise only about 2% of the normal liver population. Tumors of the liver may arise from any one of these cell types. Comparison of any one of these tumors with a whole mass of normal liver is obviously liable to be misleading because the sample of normal tissue is not completely equivalent in cell population to that of the tumor. The variable admixture of normal stromal cells found in any solid tumor, as well as such contaminants as necrotic debris, inflammatory cells, and blood may further interfere with obtaining valid biochemical information from

masses of tumor tissue. Nor does biochemical examination of the tissue at various stages in carcinogenesis get around the difficulty, for Daoust (7) has shown that there may be drastic changes in relative proportions of the various cell types during carcinogenesis.

Allard and deLamirande (8) have tried to meet these difficulties by comparing liver tumors with precancerous livers that were known to have about the same cell population distribution, i.e., 50% hepatic cells and 50% bile duct and littoral cells. Under these circumstances they demonstrated that one amino-acid-metabolizing enzyme was completely lost and two others substantially reduced in the tumor. The Montreal group have now also been able to prepare homogenates from normal and precancerous liver that consist of almost pure hepatic cells to be used for comparison with hepatomas.

Heterogeneity of a tumor is not only the result of admixture with non-neoplastic cells, but the tumor cell population itself cannot be regarded as uniform in composition either at different times in its own life history or, at any given time, from one part of the tumor to another. Isolated observations showing that tumors do not remain unchanged throughout their life history have been made over many years (9, 10, 11, 12). Foulds, adding his own similar observations, developed a general concept concerning the changes that occur with time in all tumors. He named it *progression* (13).

His concept is essentially a double one. First, it states that there are many independent heritable unit characters capable of variation in a malignant tumor. These characters include growth rate, invasiveness, ability to metastasize, morphology, function, and responsiveness to hormones. The second feature of the concept is that the characters of a tumor can change *independently* of one another and can be assorted and combined in a variety of ways. Thus a tumor may become unresponsive to hormones without a change in its growth rate, or a tumor may change its growth rate without a change in responsiveness (14).

Progression, unlike ontogenesis, is not an organized spatial and temporal development of the whole tissue. It is focal, haphazard, asynchronous, and occurs in different ways in different parts of the same tumor. Fould's own evidence for this process was based on changes that occurred with time in the histopathology of tumors and in their growth and responsiveness to hormones. As more experimental information was made available, it became obvious, as first emphasized by Klein (15) that the process of progression operates at the cellular level and not just in regions of tumors. And such new features that appeared in transplanted tumors as the ability to grow in suspension (16), to grow in genetically unrelated hosts (17), and to grow in the presence of inhibitory drugs (18) were due to spontaneous mutations occurring in the tumor cells. The nature of these mutations and whether they represent chromosomal or extrachromosomal events is not yet elucidated. It is now clear, however, that changes in hereditary character do occur spontaneously and at random in tumor cells, and these make the tumor cell population heterogeneous. This heterogeneity of cell genotype applies not only to transplanted tumors but it appears to be true of primary tumors as well. Inherited

differences from cell to cell in the chromosome number of spontaneous tumors have been demonstrated (19, 20). The fact that metastases, which represent the outgrowth of only a fraction of the primary tumor, can differ in histocompatibility characteristics (21), and in DNA content (22) from their parent tumor, suggests that inherited heterogeneity is also characteristic of primary tumors. Since the process of progression is haphazard in any tumor it also follows that whole tumors may differ from one another although they arise in the same tissue in the same animal under the influence of the same causative agent (23).

The very fact of progression with characters changing with time must also mean that the cellular composition of the same tumor will be different as the tumor grows. At its first appearance a tumor may be at any stage in progression. Nor does progression necessarily end at the death of the host, for it continues to occur if the life of the tumor is prolonged by transplantation (24). Since selective forces are always operating during the growth of these cell populations, the actual composition of any tumor at any time will depend on the selective advantage of the particular genotypes in the particular environment. This combination of mutation and selection then usually leads, sooner or later, to cells showing morphological and functional anaplasia, increased growth rate, increased invasiveness, ability to grow in the ascites form, changes in chromosome number, increase in iso- and homo-transplantability, unresponsiveness to hormones, and increased ability to resist growth-inhibitory drugs.

All this cell to cell, tumor to tumor, and stage to stage variation must have its counterpart in biochemical processes. We owe much of our present understanding of biochemical processes, and especially of the interrelationships of nucleic acids and proteins, to information obtained from microorganisms. Success in this latter area has in large measure been due to the fact that the microbiologist has avoided the pitfall of working with heterogeneous cultures. The microbiologist engaged in biochemical work with microorganisms first satisfies himself that each strain with which he is working is genotypically pure.

Since carcinogenesis usually involves outgrowth from a single cell type of a heterogeneous normal tissue, this situation is in many ways comparable to the selection of a single bacterial species from a mixed culture. Thus, if normal bile duct cells contain a very different amount of a certain enzyme than do hepatic or littoral cells, then a difference in enzyme content of a liver tumor as compared to a normal liver could be entirely due to a difference in the relative numbers of these respective cells in the liver and not to a fundamental change in enzyme content associated with neoplasia. An example of this difficulty would be the determination of ATPase levels in normal and cancerous liver (25). Novikoff (26) has shown histochemically that this enzyme is localized almost exclusively in bile duct cells. Obviously the level of ATPase in the tumor would depend on, among other things, the relative proportion of bile duct cells to other cells. The situation becomes even more complicated as a result of the changes that constantly occur during the subsequent growth

of the tumor. It can readily be seen that the resulting biochemical heterogeneity could go far beyond a single enzyme. Differences in the biochemical behavior from tumor to tumor and from primary to transplanted tumor would, therefore, be expected and have been observed (27). But, because of the confusion inherent in the heterogeneity and everchanging nature of the biological material, the meaning of these differences in terms of their relevance to malignancy must remain obscure.

Since heterogeneity is a fact, and since the eventual understanding of the cancer process lies in biochemistry, how does one face this dilemma?

One kind of biological material relatively free from the hazards of heterogeneity and useful in the study of cell biochemistry is the ascites tumor. Ascites tumors are free of stroma and of necrotic material and many of them can be grown in the animal from a single cell. In such clonal tumors a high degree of genetic and biochemical homogeneity is ensured. The ascites tumors, moreover, offer the distinct advantage of being amenable to quantitative work. Since they grow as individual and free cells in the peritoneal cavity, accurate quantitative treatment of this material is possible as shown by Klein and his co-workers and many others (28). It should not be forgotten, however, that the biochemical properties of these long transplanted tumors are probably quite different from those of any primary tumor and no satisfactory controls with normal tissue are possible. In themselves, however, they can serve as excellent models for the study of the biochemical machinery of a cell.

To approach the problem of the biochemical changes relevant to malignancy, it would be perhaps better to compare not tumor with normal tissue but to compare lines of tumor cells derived from the same original growth yet differing in a single biological characteristic such as growth rate or hormone dependence or ability to metastasize or ability to grow in the ascites form or some feature that gives the cell an advantage in its host. One could then try to correlate biochemical features with one of these biological properties that change during progression and are relevant to the process of malignancy. The difficulty will be to get pure lines from the same tumor but differing by a single biological characteristic. Purdom, Ambrose, and Klein (29) using such tumor pairs have shown differences in the surface charge and in the adhesiveness of cells that had been derived from the same tumor but which differed in their ability to metastasize, to grow in the ascites form, and to survive in minimal media. Several other pairs of cell lines are now available or can be produced experimentally. These include hormone-dependent and autonomous sublines (30), hormone-responsive and -unresponsive sublines (30), drug-resistant and drug-sensitive sublines (31, 32, 33), tumor cell lines that differ by a single histocompatibility gene (17), tumor cell lines that differ with regard to chromosome characteristics and transplantability (34), and tumor lines that grow continuously in tissue culture but which differ in virulence when transplanted to animals (35, 36). The biochemistry of progression is a relatively untouched field, and it appears to us to be a fertile one.

Although we know that the basic defect in a tumor cell involves a breakdown of growth regulatory processes leading to unrestrained division, our knowledge of how normal cell division is regulated is at most rudimentary (37). Growth control mechanisms are known which operate both between cells and within cells. One mechanism of cell-to-cell growth control is mediated by hormones, and although innumerable endocrine interrelations between organs have been described, we still know little about how these hormones act *at the cellular level.* The steroid hormone-activated hydrogen transfer system discovered by Talalay *et al.* (38) appears to be a significant recent advance in this direction. Hormone-dependent and autonomous tumor pairs could offer unique material for studying both the mechanism of action of hormones and the nature of the biochemical lesion that leads to unresponsiveness to hormones (cf. Klein (2)).

Another known mechanism of cell-to-cell interaction is the system whereby the growth of an organ and its maintenance at a certain mass are regulated through organ specific chemical mediators (cf. Weiss (39)). For instance, Ebert and Rose and others have shown that extracts of certain organs inhibited the development of the homologous organs (40, 41, 42). Also Glinos and Gey (43) have found that the serum of fully grown rats inhibited the outgrowth of liver explants in tissue culture, and the presence of a mitotic inhibitor specific for liver cells in vivo has been demonstrated in adult liver by Stich and Florian (44). It is possible that the initiation of the neoplastic process may involve a breakdown of such control processes. Again, our knowledge of such processes at the biochemical level is rudimentary at best.

The control mechanisms just discussed operate *between* different cells, i.e., secretion products from one cell control the growth of another cell. Work with microorganisms has shown that a whole series of control mechanisms exist *within* the cell. The control of the biosynthesis of enzymes in bacteria under the influence of substrates has been extensively investigated and hardly needs mention here. Inducible enzyme systems have also been demonstrated in animal cells (45). It has also been found that the bacterial cell contains systems of "feedback" control whereby the products of enzymatic reactions regulate both the activity and the synthesis of enzymes involved in the formation of these products. Gorini and Mass (46) have shown that the presence of arginine in the growth medium can completely suppress the enzyme required for its own synthesis. Umbarger (47) has recently shown in *E. coli* that the activity and the formation of the enzyme threonine deaminase is controlled by the level of isoleucine, the amino acid produced as far away as six enzymatic steps after threonine deamination.

The list of known enzymes and metabolic pathways under such feedback control is steadily increasing and it is possible that all metabolic processes intracellularly are governed by the amounts of substrate or product in many ways. Extracellular cell-to-cell interactions may also be acting in this way. A series of events involving loss or gain of various metabolites could also result from mutation or infection with a virus, and this, in turn, could completely disturb the normal enzymatic pattern of the cell. It is now known that infec-

tion of *E. coli* with T2 bacteriophage leads to the synthesis of many new enzymes and to a substantial increase in the level of other enzymes (48–50). The relation between enzyme level control and growth control is not yet clear. However, it is our feeling that information in this area is essential to our understanding of the neoplastic process, and the information required is of a biochemical nature. It may not be important where these control mechanisms are studied, but it is probable that the most fruitful systems will be those that can be studied in vitro mainly because of ease of handling, ease of quantitation, and possible selection of uniform systems.

An interesting system in which neoplasia is apparently accompanied by the synthesis or activation of new systems of enzymes is crown gall tumorigenesis in plants (51, 52). Here a tumor is induced as a result of successive treatment of the cells with wound juice, *Agrobacterium tumefaciens*, or DNA from this bacterium (53), and indole acetic acid or its physiological equivalent, each for a definite period of time and in the sequence given. Under these conditions the tumor that results grows rapidly, and is autonomous both in vivo and in vitro. The most striking feature of this process is that normal carrot cells which require 6-furfurylaminopurine, naphthalene acetic acid, glutamine, asparagine, inositol, and guanylic and cytidylic acids for optimal growth in vitro become tumor cells that can grow completely independently and have acquired the capacity to synthesize these metabolites. When any stage of the tumorigenic process is incomplete, tumors can still be obtained, but these, in contrast to tumors resulting from complete induction, require some of the metabolites for growth. Thus, under experimental control, a variety of tumors can be obtained from the same tissue which are genotypically distinct in having different nutritional requirements. Without implying that the mechanism of tumorigenesis in plants is identical with that in animals, it appears to us that the plant system offers unique opportunities for studying biochemical changes concomitant with inherited changes leading to neoplasia.

It would be very useful to the biochemist if an animal cell system amenable to the study of carcinogenesis in vitro were available. Fortunately such systems may be at hand very shortly. Temin and Rubin have shown that infection of chick embryo fibroblast in vitro with Rous sarcoma virus leads very quickly to transformation of the normal cells into tumor cells (54). In this situation one may, therefore, have a population of cells of constant genotype and uniform biochemical background, and change these into tumor cells under exactly controlled conditions. It should be possible, therefore, to study in biochemical terms the changes that occur due to infection with a virus. More and more tumor viruses are being uncovered now; it should become possible to study their action in vitro. These systems should offer at least one way to follow the process of carcinogenesis in detail in a single cell species. Admittedly this may be only one of the mechanisms whereby a normal mammalian cell is transformed into a cancer cell, but in the absence of any other clear or unequivocal knowledge of the process of carcinogenesis, the understanding of

the biochemistry of virus induction of tumors may serve as an excellent model from which to develop concepts of the whole process of carcinogenesis.

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